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Dissertação

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"Don't bother just to be better than your contemporaries or predecessors. Try to be better than yourself."

William Faulkner

Abstract

Cell-to-cell communication or quorum sensing is an important bacterial process of intercellular communication that enables bacteria to determine their numbers by producing, secreting and detecting the accumulation of signalling molecules, called autoinducers. This process allows bacterial populations to synchronize gene expression and coordinate, at the group level, complex behaviours such as antibiotic production, biofilm formation or virulence.

Pectobacterium carotovorum are Gram-negative bacteria which inhabit the soil and infect vegetables, causing large economic problems. Production of virulence factors in this specie is tightly regulated by two quorum sensing mechanisms: ExpI/ExpR system which uses the N-acyl homoserine lactone signal; and the GacSA/Rsm system in which the signal is unknown. Ecc15 is a strain of *P. carotovorum* that is able to persist in the gut, when oral ingested, by *Drosophila melanogaster* larvae. This persistence ability was attributed to the acquisition of a gene denominated *Erwinia* virulence factor (*evf*). Although the host immune response to this bacterium was extensively studied, the effect at the host development level upon bacterial infection was never addressed. Additionally, the genetic network controlling *evf* expression and the Evf specific mechanism of action in the gut remains poorly understood.

In this work we studied the effect of the exposure to Ecc15 in the host development by following the time to pupation of larvae exposed to bacteria. We also investigated the Evf specific mechanism of action in the context of the host-microbe interaction by testing the effect of bacterial free supernatants and purified Evf protein. Moreover, we investigated the genetic network regulating *evf* transcription by monitoring the expression of the promoter regions of the known and putative regulators of *evf* (*hor* and *evr*, respectively). Finally we studied the role of quorum sensing and *evf* possible regulators in this specific interaction by exposing larvae to mutants in these genetic systems.

In conclusion we observed that larvae exposed to wild-type Ecc15 have a development delay of approximately one day and that this effect is *evf*-mediated. We concluded the *evf*-effect is caused by interruption of feeding behaviour and that the product of the *evf* gene is not extracellularly toxic. In addition, we optimized a method to construct mutants in Ecc15 that allowed us to elucidate part of the genetic network involved in regulation of *evf.* Lastly we showed the role of the quorum sensing in the *evf*-mediated effect of Ecc15 in *Drosophila* development *in vivo*

Keywords: quorum sensing; *evf*; *Drosophila* development; microbe-host interactions; bacterial signaling; bacterial pathogenesis.

Resumo

Na maior parte dos casos, em ambientes naturais, os microrganismos fazem parte de uma comunidade grande e diversa. Assim como os Humanos desenvolveram a linguagem em resposta à necessidade de comunicação entre indivíduos, os cientistas descobriram que as bactérias também são capazes de comunicar umas com as outras através de sinais químicos. A comunicação célula-a-célula, ou *quorum sensing*, é um importante processo bacteriano que permite às bactérias determinar a sua densidade populacional através da produção, secreção e deteção de moléculas sinalizadoras chamadas auto-indutores. Este processo permite que as populações bacterianas sincronizem a expressão de genes e coordenem complexos comportamentos de grupo, como a produção de antibióticos, formação de biofilmes ou virulência.

Pectobacterium spp. são bactérias Gram-negativas que habitam o solo e causam a doença da podridão mole em legumes. Estes agentes fitopatogénicos infetam diversos vegetais tais como batatas, cenouras ou aipos, causando importantes perdas económicas e de produção agrícola. A virulência desta bactéria está associada à produção de enzimas que degradam a parede celular. Estas enzimas são produzidas em elevadas quantidades e secretadas para o meio extracelular, resultando na maceração dos tecidos vegetais. Apesar do potencial virulento destas enzimas, a sua produção implica grandes custos energéticos para as bactérias. Portanto, de forma a serem efetivas, a sua produção é altamente regulada por dois sistemas de *quorom sensing*: o sistema ExpI/ExpR que usa as homoserinas lactonas aciladas como moléculas sinalizadoras e o sistema GacSA/Rsm cujo sinal é ainda desconhecido.

O sistema ExpI/ExpR é um sistema de *quorum sensing* do tipo LuxI/LuxR, composto por uma sintetase de homoserinas lactonas ExpI e por dois recetores ExpR1 e ExpR2. As homoserinas lactonas têm a particularidade de se difundir livremente através da membrana e a sua concentração depende da densidade populacional. A baixas densidades celulares, quando a concentração de homoserinas lactonas é baixa, os recetores ExpR1 and ExpR2 ligam-se à região promotora da proteína reguladora RsmA estimulando a sua expressão. Esta proteína liga-se ao RNA de vários alvos, incluindo o das enzimas que degradam os componentes da parede celular vegetal, promovendo a sua degradação e reprimindo a virulência. Num cenário de altas densidades celulares, quando a concentração de homoserinas lactonas aumenta, estas ligam-se aos recetores, levando à restrição da interação DNA-proteína, que resulta na diminuição da expressão de *rsmA* e assim promovendo a expressão dos genes associados à virulência.

O sistema GacSA/Rsm é um sistema canónico de dois componentes. É composto pela cinase sensora GacS e pelo regulador de resposta GacA. Apesar do sinal que despoleta a ativação deste sistema ser desconhecido, sabe-se que a ativação é dependente da densidade celular. Num cenário de alta densidade celular, a cinase GacS fosforila o regulador de resposta GacA promovendo a transcrição do RNA não codificante *rsmB*. Este sRNA liga-se à proteína RsmA inativando-a, o que resulta na ativação da expressão de genes relacionados com virulência.

Assim, o sistema ExpI/ExpR e o sistema GacSA/Rsm coordenam a produção de fatores de virulência, como as enzimas que degradam componentes da parede celular vegetal, através da regulação dos níveis de RsmA e *rsmB*.

Ecc15 é uma estirpe da espécie fitopatogénica *Pectobacterium carotovorum* que tem a particularidade de persistir no aparelho intestinal de *Drosophila melanogaster* quando ingerida oralmente. Esta capacidade foi atribuída à aquisição de um único gene denominado *evf* (*erwinia virulence factor*). Juntamente com este gene, dois reguladores foram identificados: *hor* uma proteína da família dos reguladores SlyA, e *evr* (*erwinia virulence regulator*) cujo papel na expressão de *evf* nunca foi investigado.

Foi também descrito que a persistência da estirpe Ecc15 no intestino das larvas de *Drosophila* é um fenómeno transitório, em que a bactéria é eventualmente eliminada pelo sistema imunitário da larva. No entanto, numa fase crítica do desenvolvimento dos insetos, como é a fase larvar, uma infeção provoca o deslocamento dos recursos nutricionais de acumulação de reservas para ativação do sistema imunitário, o que pode ter consequências ao nível do desenvolvimento do hospedeiro. De facto, apesar de a resposta imunitária à infeção transitória causada pela estirpe Ecc15 ter sido intensamente estudada, a consequência desta para o desenvolvimento do hospedeiro nunca foi avaliada.

Neste trabalho, propusemo-nos a estudar a rede genética que regula a transcrição do gene *evf* e o papel do *quorum sensing* na interação microrganismo-hospedeiro, com base na hipótese de que a expressão de *evf* é regulada por *quorum sensing* usando os componentes envolvidos na regulação da produção de factores de virulência associados à degradação das plantas. Para além disso, investigámos o efeito da exposição bacteriana no desenvolvimento do hospedeiro e o mecanismo de acção específico do Evf ao nível desta interação

De modo a investigar o efeito da exposição à bactéria Ecc15 ao nível do hospedeiro, monitorizámos o desenvolvimento das larvas ao longo do tempo, em específico a passagem para o estádio de pupa, após infeção com Ecc15. Os resultados mostraram que larvas infetadas com a estirpe *wild type* (wt) de Ecc15 tinham um atraso no desenvolvimento, com apenas 50% das larvas a progredirem para pupa ao fim de 2 dias, em oposição às larvas controlo

(alimentadas só com comida de mosca) com mais de 90% a atingirem o estado de pupa no mesmo período de tempo. Por outro lado, larvas infetadas com uma bactéria mutante no gene *evf* não mostraram qualquer atraso no desenvolvimento (93% tinham pupado ao dia 2) quando comparado com as larvas infetadas com a bactéria wt. Estes resultados revelam que a infeção transiente da bactéria Ecc15 provoca um atraso no desenvolvimento do hospedeiro e que este atraso é dependente do gene *evf*.

Tendo em conta que a paragem de ingestão de comida é uma resposta comum durante uma infeção e que este comportamento pode causar problemas ao nível do desenvolvimento, monitorizámos a quantidade de comida ingerida pelas larvas após exposição à bactéria. As larvas foram infectas com a respetiva bactéria e postas em tubos contendo comida de mosca corada. A quantidade de comida ingerida foi inferida, quantificando a porção de pigmento azul ingerido 3 e 22 horas após infeção. Os resultados mostraram que 3 horas após o tratamento as larvas infetadas com a bactéria wt tinham ingerido pouca ou nenhuma comida quando comparado com as larvas infectas com o mutante *evf* ou com o controlo. Passadas 22 horas após infeção, pudemos observar pigmento nas larvas infetadas com a bactéria wt indicando que as larvas tinham retomado o consumo de comida. Estes resultados sugerem que a infeção que provoca o comportamento anormal das larvas foi eliminada, permitindo-lhes recomeçar a ingestão de comida de modo a prosseguir o desenvolvimento.

Apesar de ter sido anteriormente postulado que o gene *evf* altera a fisiologia normal do hospedeiro cessando os movimentos peristálticos normais do intestino, o mecanismo específico pelo qual *evf* produz este efeito permanece desconhecido. Para perceber se o mecanismo de acção da proteína Evf ao nível do hospedeiro estaria relacionado com toxicidade extracelular, testámos sobrenadantes de uma cultura *overnight* e proteína Evf purificada no desenvolvimento das larvas. Foram analisados sobrenadantes de culturas wt, mutante *evf*, e Ecc15 wt a sobreexpressar o gene *evf*. Os resultados revelaram que nenhum dos sobrenadantes teve efeito ao nível do desenvolvimento larvar, com todas as larvas a comportaram-se de forma similar às larvas controlo. Suportando os dados dos sobrenadantes, a aplicação da proteína purificada também não teve um efeito relevante no desenvolvimento do hospedeiro. Estes resultados indicam que se a proteína Evf é uma toxina não é activa no meio extracelular, sendo provavelmente injetada no hospedeiro.

Para estudar a rede genética envolvida na regulação da expressão do gene *evf* construímos, pela primeira vez, mutantes na estirpe Ecc15. Para isso usámos uma adaptação do método de recombinação descrito por Wanner e Datsenko. Escolhemos, como sendo os genes relevantes para compreensão da rede regulatória do *evf,* os genes *expI* e *gacA* como parte integrante da regulação por *quorum sensing*, e os genes *hor* e *evr* por serem conhecidos

como reguladores de virulência. Sendo Ecc15 uma estirpe patogénica de plantas, os mutantes foram caracterizados para a expressão de fatores de virulência associados à degradação de componentes vegetais. Para isso usámos um protocolo para medir a maceração de batatas. Ambos os mutantes nos genes de *quorum sensing* (*expI* e *gacA*) apresentaram níveis de maceração muito baixos quando comparado com a bactéria wt, tal como os mutantes nos genes *hor* e *evr*. Curiosamente, o mutante *evf* apresentou níveis de maceração semelhantes ao wt. Estes resultados revelaram a importância do *quorum sensing* na expressão de genes associados à virulência em infeções de vegetais nesta estirpe bacteriana. Para além disso, mostraram que os genes *hor* e *evr* desempenham um papel na produção destes fatores ou em genes associados à sobrevivência na batata. Contrastando com a infeção das larvas, os resultados mostraram que o gene *evf* não tem qualquer papel na expressão dos fatores de virulência envolvidos na degradação das plantas, o que suporta a hipótese de aquisição génica especifica para a interação com a mosca da fruta.

De forma a avaliar se a expressão do gene *evf* seria também regulada por *quorum sensing* fizemos fusões transcricionais com GFP (*Green Fluorescent Protein*) do gene conhecido como seu regulador (*hor)* e do regulador putativo (*evr*) e avaliámos a sua expressão ao longo do tempo. Para isso usámos citometria de fluxo e inferimos a expressão destes genes medindo a quantidade de GFP por célula em cada um dos mutantes. Os nossos resultados revelaram que num mutante *expI* a expressão génica (mais evidente no gene *hor*) está diminuída. Esta pode ser complementada adicionando homoserinas lactonas de forma exógena, o que é característico de regulação por *quorum sensing*. No entanto, os resultados também mostraram que o perfil de expressão de ambos os genes é diferente do perfil padrão de um gene regulado por *quorum sensing*. A expressão destes genes atinge o seu pico a meio da fase exponencial (4 horas de crescimento), contrastando com o observado normalmente em genes regulados por *quorum sensing* onde os picos de expressão ocorrem na fase estacionária. Os nossos resultados mostram que o *quorum sensing* tem um efeito na expressão destes genes *in vitro*, uma vez que a sua expressão é alterada na presença de homoserinas lactonas. No entanto, contrasta com a típica regulação por *quorum sensing*, já que na fase estacionária a expressão é reprimida por um mecanismo desconhecido.

Por último, avaliámos o papel do *quorum sensing* e dos reguladores do gene *evf* no contexto do desenvolvimento *in vivo*. Para isso infetámos as larvas com os respetivos mutantes usando o protocolo descrito anteriormente. Em relação aos mutantes em genes de *quorum sensing*, os resultados mostraram que larvas infetadas com o mutante *expI* não tiveram qualquer atraso no desenvolvimento (87% de pupas ao dia 2) evidenciando uma dinâmica similar às larvas controlo. Por outro lado, as larvas infetadas com o mutante *gacA*

apresentaram um fenótipo parcial, com um atraso no desenvolvimento (53% de pupas ao dia 2) significativamente diferente do controlo mas também do wt. Quanto aos mutantes nos genes reguladores do *evf,* larvas infetadas com o mutante *hor* não apresentaram qualquer atraso no desenvolvimento. Contrastando com este resultado, larvas infetadas com o mutante *evr* exibiram um fenótipo semelhante ao wt, com apenas 42% das larvas a atingirem o estádio de pupa ao dia 2. Isto revela que provavelmente este gene não tem um papel importante na expressão do gene *evf*. Coletivamente, estes resultados mostram que os genes *expI* e *hor* têm um papel importante no efeito causado por *evf*, assim como gene *gacA* parece ter uma função *in vivo*. No que toca ao gene *evr* os resultados indicam que não parece ter um papel relevante no efeito mediado por *evf*.

Em suma, este trabalho elucidou o efeito da infeção transiente da bactéria Ecc15 no desenvolvimento da larva de mosca da fruta. Mostrámos que o efeito é causado por interrupção do comportamento alimentar normal e que o produto do gene *evf* não é toxico, pelo menos ao nível extracelular. Otimizámos ainda um método de construção de mutantes por recombinação homóloga em Ecc15 que nos permitiu elucidar parcialmente a rede genética reguladora da expressão do gene *evf*. Por fim mostrámos ainda o papel do *quorum sensing in vivo*, embora não tenha ficado completamente esclarecido se o efeito é direto, afetando o gene *evf* ou indireto, comprometendo a capacidade da bactéria permanecer e sobreviver no interior do hospedeiro.

Palavras-chave: *quorum sensing*; *evf*; Desenvolvimento de *Drosophila*; Interacções microrganismo-hospedeiro; Sinalização bacteriana; Patogénese bacteriana;

Index

1. Introduction

1.1 Quorum sensing: the language of bacteria

Quorum sensing (QS) is a process of intercellular communication that enables bacteria to determine their own numbers by producing, secreting and detecting the accumulation of signalling molecules[1,2], also called autoinducers. These molecules accumulate in the extracellular environment as bacteria multiply and when a threshold concentration is reached, the signal is detected and changes at the level of gene expression of the population occur[2]. This way, bacteria are able to coordinate gene expression and synchronise complex group behaviours across the population, such as antibiotic production, biofilm formation, expression of virulence factors, sporulation or competence[3].

The first QS system described was the Lux system[4,5] of *Vibrio fisheri* and is present in a large number of Gram-negative bacteria. This marine bacterium uses this system to coordinately activate the expression of the *lux* operon, which leads to the production of light also known as bioluminescence. The Lux system is composed of an acyl-homoserine lactone (AHLs) synthase, LuxI, and a cytoplasmic receptor, LuxR. At high cell densities the threshold concentration of AHLs is reached and LuxR binds to the signal molecules. This causes a conformational change in LuxR which will act as a transcription regulator inducing the expression of the *lux* operon. This operon includes the genes responsible for expressing luciferase which is responsible for producing bioluminescence. This process is essential for the symbiotic interaction of *V. fisheri* with its natural host, *Euprymna scolopes,* the bobtail squid[6]. Bacteria colonizing the squid light organ produce light that is used by the squid to better escape from its predators. In exchange, the squid provides a nutrient rich environment for the bacteria. It was shown, using *V. fischeri* mutants, that light production and the ability to colonize are tightly related[7]. *V. fischeri* mutants affected in the genes responsible for light production or QS components display impaired colonization of the light organ. The enzyme luciferase catalyses a chemical reaction in which one of the unstable intermediates emits light[8]. It was shown that mutants in the synthesis of luciferase are impaired in the ability to persist in the light organ. Likewise, QS mutants affected in the signal synthase, LuxI, or in its receptor, LuxR, are also unable to produce light and similarly to what was observed for the luciferase mutants, QS mutants are also unable to establish a symbiotic relationship with the squid.

Gram-negative bacteria are not the only organisms regulating behaviours in a density-dependent manner. Gram-positive bacteria use a similar system where small peptides are used

as signals instead of AHLs. These peptides are secreted to the extracellular media where they can be detected by a two-component system comprising a sensor kinase and a response regulator[9,10]. The resulting signalling and changes in gene expression leads to changes in group behaviours. For example the production of virulence factors in *Staphylococcus aureus* is regulated by the Agr QS system, a typical Gram-positive QS mechanism[9].

Although the two mechanisms presented here are species-specific, there is also a third, the autoinducer-2 (AI-2) system, which has been proposed to act as an interspecies cell-to-cell communication system. The AI-2 signal is produced by the LuxS synthase and can be recognized by both Gram-positive and Gram-negative bacteria[11]. It has been shown to control a variety of group behaviours, such as biofilm formation (*Bacillus cereus*), motility (*Helicobacter pylori)* or bioluminescence (V*ibrio harveyi*)[12].

Though QS has been studied for five decades, proper *in vivo* models to study its function in host-microbe interactions are still limited. Although it has been shown that QS signals and regulators are essential for activating functions that are necessary for microbe-host interactions (bioluminescence), the benefit of regulating those in a density-dependent manner has not been formally demonstrated. Indeed, it has been postulated that QS dependent regulation might have a role in regulating complex host-microbe interactions such as transmission to new hosts and gut colonization but experimental models to test this hypothesis are missing.

One group of bacteria that relies on QS to precisely synchronise the production of their virulence factors is the *Pectobacterium* spp. These bacteria are Gram-negative *Enterobacteriaceae* which inhabit the soil and cause soft rot disease. These plant pathogens can infect several vegetables, such as potatoes, carrots or celery, causing important agricultural and economic losses. The virulence of these bacteria is associated with the production of plant cell wall-degrading enzymes (PCWDEs)[13,14]. High levels of these important enzymes are secreted into the extracellular milieu, resulting in general plant tissue maceration. Known PCWDEs include proteases, cellulases and pectinases, which convert cell wall components into nutrients that can be used by bacteria. Pectinases are the most relevant to disease development. These include pectin lyases, pectate lyases and polygalacturonases, all of which break down pectin, an essential component that maintains the structure of the plant cell wall by creating a matrix that involves the cellulose-hemicellulose network. Despite the virulence potential of PCWDEs, their production comprises great energy costs to bacteria and in order to be effective they are activated only when the optimum conditions are met. Therefore, the production of these enzymes in *Pectobacterium* spp. is tightly regulated by QS.

The phytopathogenic *Enterobacteriaceae Pectobacterium carotovorum* (formerly *Erwinia carotovora*) strain Ecc15, has been shown by Bruno Lemaitre and colleagues as being capable of persisting in the gut of *Drosophila melanogaster* and might be using this insect as an transmission vector. Given the tractability of this model system both from the microbe and the host side we decided to investigate the role of QS in this specific interaction. The *Pectobacterium-Drosophila* interaction allows us to study the importance of QS in bacterial infections *in vivo* and ultimately explore the possible role of QS in transmission between hosts.

1.2 The QS system of *Pectobacterium carotovorum*

P. carotovorum strain Ecc15 was first isolated in 1978 from an infected potato field in France[15]. Despite its economical relevance, no studies were performed to understand its signalling networks regulating virulence, though the closely related strain Ecc71 has been studied in detail[16–18] and the regulation of virulence in these two strains was assumed to be identical. Moreover, other *Pectobacterium* spp. like *P. wasabiae* or *P. atroseptica* have been extensively studied and their signalling networks shown to be very similar to *P. carotovorum*. The production of PCWDEs in *Pectobacterium* spp. is regulated by two QS systems: an AHLs system[17,19] and the GacSA/Rsm system[16] (Figure 1). The AHL system, a LuxI/LuxR type QS system, is the most studied communication system in *Pectobacterium.* It is composed by ExpI (LuxI homolog) that synthesizes AHLs, which diffuse across the membrane to the extracellular environment, and by two receptors, ExpR1 and ExpR2[18,20] (LuxR homologs). At low cell densities (low concentrations of AHLs) these receptors bind to the promoter region of the RNA-binding protein, RsmA[21], leading to an increase of its expression[22]. RsmA binds to several targets, such as the mRNAs of PCWDEs, promoting their degradation and repressing virulence. Interestingly, the ExpR1 and ExpR2 regulators can only bind DNA in absence of AHLs, contrasting to what is observed in the majority of the LuxI/LuxR type QS systems[23]. At high cell densities, AHLs are recognized by ExpR1 and ExpR2, restricting binding to the *rsmA* promoter, thus stopping activation of *rsmA* transcription, which results in the relief of RsmA-mediated repression of virulence and increasing expression of virulence genes like PCWDEs. As in the LuxI/LuxR systems, at high cell densities, high concentration of AHLs also regulates activation of virulence.

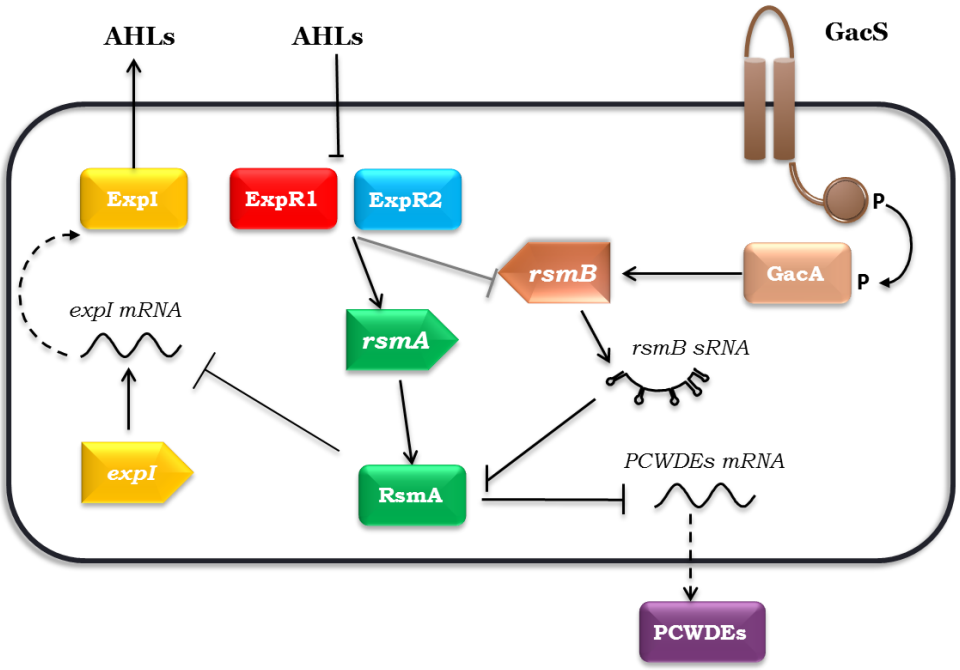

Figure 1 –Quorum Sensing network for the regulation of plant virulence in *Pectobacterium* spp. At low cell densities (low levels of AHLs), ExpR1 and ExpR2 bind to *rsmA* promoter region inducing its transcription. RsmA binds to the PCWDEs mRNA promoting its degradation and consequently repressing virulence. At high cell densities the AHLs are at high concentration and bind to the two receptors, which no longer promote the expression of *rsmA* leading to derepression of virulence. Additionally, the GacSA/Rsm system is activated and promotes the expression of *rsmB* which binds to the existent RsmA proteins blocking their action and thus also promoting expression of the virulence factors.

The GacSA/Rsm is the second system which controls the expression of PCWDEs[16]. This canonical two component system works together with the ExpI/ExpR system to control the expression of PCWDEs. The GacSA/Rsm is homologous to the GacSA/Rsm two component system from *Pseudomonas aeruginosa* and the BarA/UvrY/Csr system from *Escherichia coli*[24]. It is composed by a sensor kinase GacS and a response regulator GacA. Although the sensor is activated by an unidentified signal, it is known that the activation is regulated in a density dependent manner. At high cell density, upon activation, GacS phosphorylates the response regulator GacA, promoting the transcription of the small non-coding RNA *rsmB*[25]. This sRNA binds RsmA and acts as a competitor with RsmA targets. RsmA sequestration by *rsmB* results in inactivation of RsmA and derepression of virulence[26]. Recently, it has been shown that *rsmB* expression is not only controlled by the GacS/GacA system but also by the ExpI/ExpR system (unpublished data from our laboratory). At low cell densities, *rsmB* expression is inhibited and *rsmA* transcription is promoted by ExpR1 and ExpR2, upregulating the RsmA levels and consequent repression of virulence. Together, the ExpI/ExpR and the GacSA/Rsm systems control the production of PCWDE's by regulating RsmA. At high cell density, AHLs promote inhibition of *rsmA* transcription while *rsmB* acts via a post-translational mechanism to inhibit RsmA by sequestration.

The integration of these two cell-to-cell communication systems tightly controls the expression of these virulence factors, which are essential for the success of bacteria in their natural environment. Presumably, by using QS systems, bacteria can ensure that virulence factors are only expressed when they are effective, highlighting the relevance of these systems in the bacterial world.

1.3 *P. carotovorum* and *Drosophila* - a host-microbe interaction

How *Pectobacterium* spp. are transmitted between their plant hosts has been a question intriguing scientists since 1980. The first obvious thought was that, being a phytopathogen, it survives in the soil and infects injured plants. Though, studies performed in the last century showed a rapid decline of the population size in the soil[27], and even though the presence of plant material in the soils (e.g. the remains of the harvesting) enhanced the resistance of these bacteria to the soil conditions, promoting survival[27], it was concluded that in general *Pectobacterium* spp. are present in the soil in amounts below detection levels but can expand their numbers when exposed to plant materials. However, invasion of new sites and the routes of transmission to new hosts are still not understood. In 1981 it was proposed by J.W.Kloepper, J.W.Brewer and M.D.Harrison that insects from the *Drosophila* family could act as vectors of *Pectobacterium* spp[28] as occurs with many other pathogens, including those of human. They suggested that transmission could take place by three non-specific interactions: carriage on the body, regurgitation and defecation. Although these are valid hypothesis, most likely these interactions are not passive, as previously assumed. In fact, nowadays, most researchers think dissemination of pathogens by vectors involves specific interactions between the host and the bacteria[29]. Supporting this hypothesis, it has been shown that insects have sensitive mechanisms to recognize microbes and mediate interactions with these organisms, relying upon multiple innate immune responses that are shared with higher organisms[30].

Drosophila melanogaster, commonly known as fruit fly, is a model organism to study immunity and responses to pathogens[31,32]. Due to the similarities between the innate immune system of *D. melanogaster* and that of mammals, this insect is considered to be a good model to study host-microbe interactions[33]. In addition to the presence of the innate immune system, other *Drosophila* characteristics such as the short life cycle, the availability of genetic tools and easy maintenance in the laboratory make this insect an excellent model to study bacterial-host interactions and identify mechanisms to fight infections. *D. melanogaster* is highly resistant to microbes; living in an environment rich in fungi, bacteria and viruses, this insect has developed efficient ways to kill pathogens[34]. The lack of adaptive immune system does not seem to be a

problem in the insect world and they depend on their innate immunity to fight against pathogens[35]. The *Imd* and the *Toll* cascades are the two main regulatory pathways controlling immune responses in flies[30]. The Imd pathway acts upon recognition of Gram-negative bacteria whilst the Toll pathway responds to both Gram-positive and fungi recognition (Figure 2). Peptidoglycan recognition proteins (PGRPs) that recognize different forms of peptidoglycan (PGN) are present in both cell membranes and hemolymph of flies. PGN from Gram-positive and -negative bacteria differs mostly in two features: composition of the peptide chain and structure of the PGN membrane. At the third position of the peptide chain Gram-negative bacteria have a meso-diaminopimelic acid (DAP) molecule instead of the lysine residue (Lys) that exists in Gram-positive. On the other hand, Gram-negative have a single layer of PGN hidden in the periplasmic space, whereas the PGN of Gram-positive bacteria is multilayered and exposed at the bacterial surface. The Toll pathway is activated by Lys-type PGN and induces the synthesis of several peptides, such as drosomycin. DAP-type PGN activates the Imd pathway, inducing the production of different AMPs such as diptericin[36]. This production of AMPs can be systemic as well as locally associated with a tissue. Usually, in local immune responses, the first line of defence is the production of reactive oxygen species (ROS) while the production of AMPs comprises a second line of defence. Thus, in the context of an oral infection, to survive in the gut, the invading pathogens have to initially resist to ROS- and then to AMPs-mediated death[34].

Although the immune response is critical for survival, its action against foreign microorganisms is energetically costly[37–39]. In order to successfully eliminate pathogens the host has to produce a battery of antimicrobial peptides, shifting energy utilization to activation of *Toll* or *Imd* pathways[37], instead of growth and substrates storage. In *Drosophila*, the fat body is the central organ responsible for synthesis and secretion of antimicrobial peptides[40] as well as primary storage of energy sources. During an infection/inflammation, lipids metabolism is altered, leading to inhibition of storage and activation of hydrolysis of triglycerides in order to provide enough energy for immune reactions[39,41].

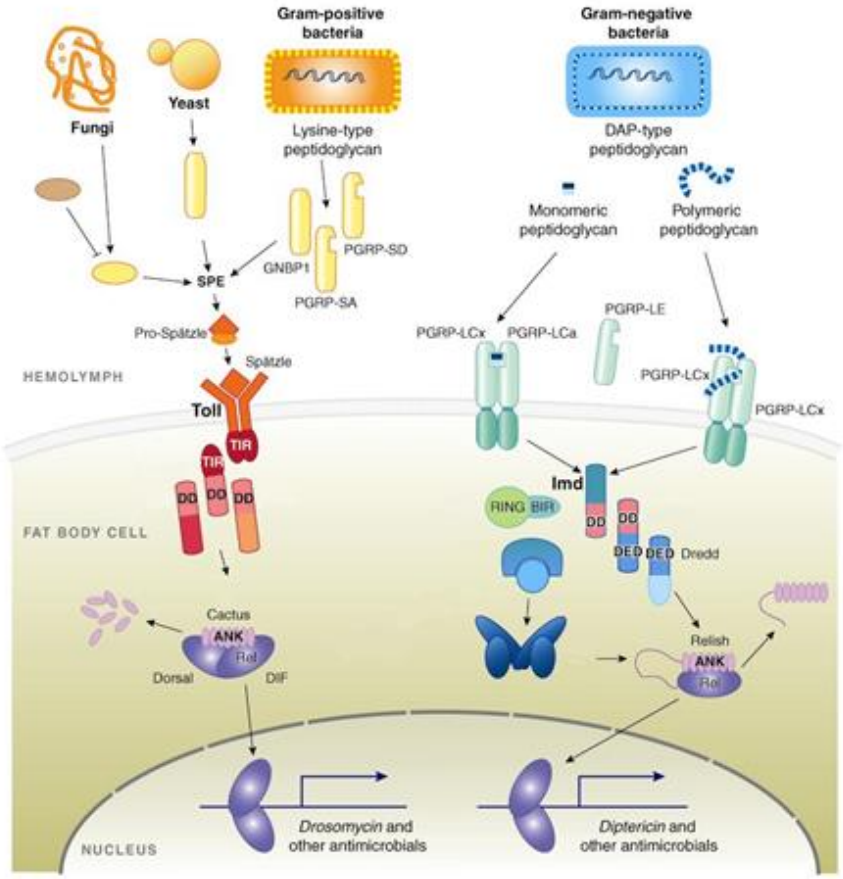

Figure 2 – Schematic representation of Toll and Imd pathway activation in *Drosophila* spp. Production of antimicrobial peptides occurs upon recognition of potential pathogenic organisms. The Toll pathway (top left) is largely activated by fungi and Gram-positive bacteria whereas Imd pathway (top right) is activated by Gram-negative bacteria. Both pathways possess membrane receptors which sense bacterial components such as the peptidoglycan. The Toll receptor is activated by a cleaved form of the protein Spatzle processed by recognition molecules (PGRP-SA, PGRP-SD or GNBP1) involved in identification of Gram-positive bacteria and fungi cellular components. This mature form of Spatzle binds to Toll[42] as dimer leading to series of signalling cascades concluding in cactus degradation[43]. This event leads to the release and reallocation of the Rel transcription factors Dif and Dorsal from the cytoplasm to the cell nucleus[44]. The allocation promotes the expression of AMPs such as Drosomycin. The Imd receptor PGRP-LC directly binds to DAP-type peptidoglycan. This interaction promotes the recruitment of the caspase Dredd[45]. This caspase associates with a phosphorylated form of Relish and cleaves it[46,47] realising the Rel domain. This domain is translocated to nucleus and promotes the expression of AMPs such as Diptericin. (Adapted from [30]).

The majority of insects have development checkpoints that need to be reached in order to continue the development process. The body size and growth rate are two essential parameters tightly controlled in insects and higher organisms through the development process[48,49]. Body size is regulated by genetic, physiological and environmental factors such as temperature, population density and nutrition[49]. It was found that insulin signalling, and in particular *Drosophila* insulin-like peptides (DILPs) are crucial for normal growth, acting as mediators between nutrition and cell growth[50]. DILPs activate *Drosophila* insulin receptors (dInR)[51,52] which trigger a series of signalling cascades, leading to production and accumulation of the cell signal phosphatidylinositol 3,4,5-triphosphate (PIP_3) which promotes accumulation

of nutrients and cell growth[53]. The fat body responds to nutrients availability and produces an unknown growth factor that regulates DILPs/dlnR signalling controlling the growth rate[54].

During development there are several size assessment events that determine if a larva is ready to enter metamorphosis or whether more growth is necessary. For example, in order to enter pupation, last stage larvae need to surpass both minimal viable weight (MVW) and critical weight (CW). MVW is defined as the minimal fat body mass needed to survive through metamorphosis[49]. CW is the development switch in which no more growth is necessary to commit to metamorphosis[55]. In *Drosophila*, contrary to other insects, these two development checkpoints appear to occur nearly at the same time, around 70 hours after egg laying[56], and thus were considered the same for a long time. However, experiments done in 1938 by Beadle and co-authors reported that larvae starved before 70 hours of development were unable to continue metamorphosis whereas larvae starved after that period entered metamorphosis but originated smaller adults[57]. It was also observed that larvae starved before reaching CW had a delay in their development for a period equivalent to the starvation period[57]. When CW is reached, an endocrine cascade for metamorphosis begins in which the titers of juvenile hormone (JH) decrease and lead to the release of the prothoracicotropic hormone (PTTH). PTTH acts on the prothoratic gland stimulating the synthesis of the hormone ecdysone. In the absence of JH, ecdysone induces metamorphosis and temporary suspension of feeding.

In a scenario of bacterial infection, a common physiological response is feed ceasing, larvae enter in starvation, and allocate resources to the activation of the immune system. This behaviour produces changes at the endocrine level which can lead to development delays[37,58,59].

Recently, two strains of *P. carotovorum*, Ecc15 and Ecc1488, were shown to trigger an immune response in *D. melanogaster* by oral ingestion, causing a non-lethal transient infection[15]. Presumably, this immune response is due to the ability of those bacteria to persist in the gut of *D. melanogaster* larvae. Importantly, a single gene present in these two *P. carotovorum* strains, was shown to be essential and sufficient to this phenotype[60]. This gene was named *evf*, standing for *Erwinia* virulence factor. Additionally, another gene, *hor,* was identified as an *evf* regulator[60]. The gene *hor* encodes a transcriptional regulator from the SlyA family and besides regulating *evf* it also shown to regulate production of antibiotics and production of extracellular enzymes in other bacteria[61]. *evf* seems to be strain restricted, it was found only in the Ecc15 and Ecc1488 *P. carotovorum* strains, while *hor* is present in many *P. carotovorum* and also in human pathogens that live in close interaction with insects, such as *Yersinia* or *Serratia* spp.[61]. Thus the described interaction of strains Ecc15 and Ecc1488 with *D. melanogaster* seems to be the result of *evf* acquisition, which presumably enables gut

persistence. An event of single acquisition dramatically changing bacterial life style is not new: acquisition of the gene *ymt* (*Yersinia* murine toxin)[62] allowed *Yersinia pestis* to colonize and proliferate in the gut of fleas turning this bacterium in one of the worst plagues in human history.

Although it was demonstrated that *evf* improves bacterial survival rate in the Ecc15-*Drosophila* model, the mechanism behind this phenomenon is not clear. In 2007, Muniz *et al.* showed that Ecc15 *evf* mutants were unable to persist in the gut of the *imd* deficient larvae. Thus it does not seem that *evf* influences directly the Imd-dependent immune response. They also hypothesized that Evf could be acting extracellularly since co-infections with Ecc15 wt and *evf* mutant allowed the mutant to persist in the gut. However, they could not detect Evf extracellularly and showed that Evf was present in the cytoplasm. Lastly, by removing the gut from the larvae body, *evf* mutants were able to persist in the same titers as the wt bacteria. They concluded by proposing that Evf might be affecting the normal gut physiology by antagonizing the peristaltic movements and thus avoiding natural bacterial elimination. Although this still remains a plausible hypothesis, no mechanism has been proposed for how Evf could have such effect. Additionally, the regulatory pathway controlling Evf production still remains to be elucidated. It was shown that Hor controls the expression of *evf*[60] and, in other *Pectobacterium* species, *hor* is regulated by QS via RsmA[63]. However, it was never evaluated if *evf* regulation by *hor* is independent or dependent of other putative regulators such as *evr*. This gene was described in NCBI gene database as a putative regulator of *evf* (http://www.ncbi.nlm.nih.gov/nuccore/AY167733.1) but no paper was published reporting its function in the regulation cascade.

Given the known role of QS in regulating *hor* in other bacterial species and its importance in regulating virulence in this strain, we hypothesize that in Ecc15 Evf production is regulated by QS by the same genetic pathway as the PCWDEs (Figure 3). Therefore, we proposed to study the regulation network of *evf* production.

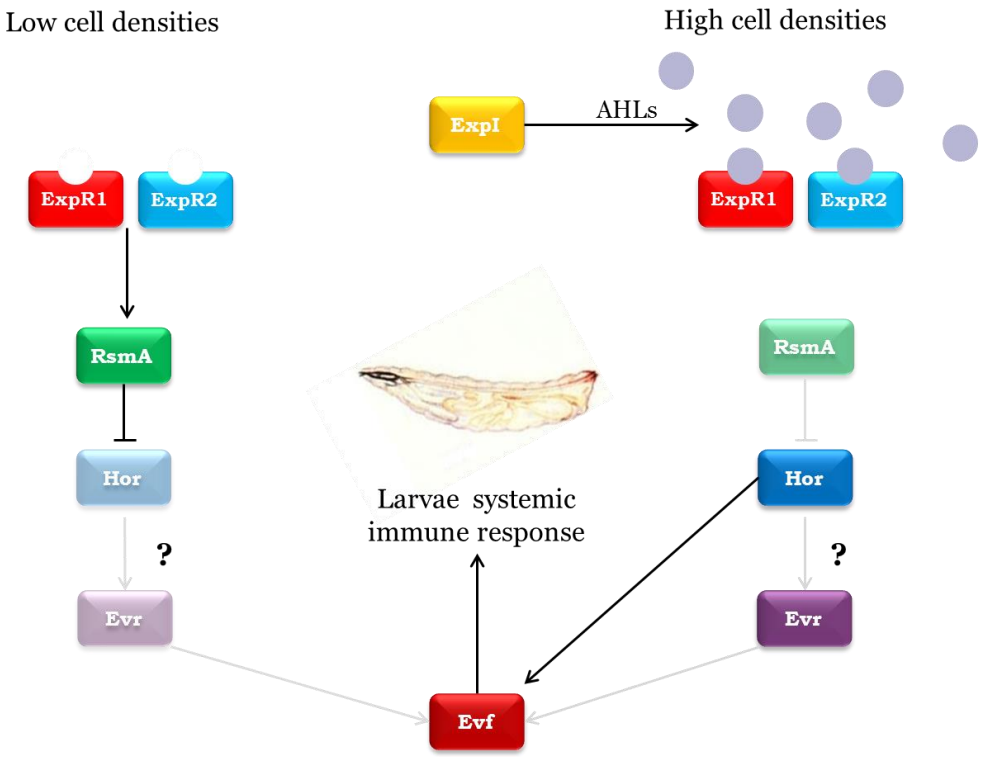

Figure 3 – Propose model for regulation of Evf production via QS. At low cell densities, ExpR1 and ExpR2 bind to *rsmA* promoting its transcription. RsmA binds to the *hor* mRNA blocking its expression. If *hor* regulates *evf* transcription then this would result on a decrease of *evf* transcription at low cell densities. At high cell densities AHLs bind to ExpR receptors, and these no longer can activate transcription of *rsmA*. RsmA protein levels decrease and Hor protein levels increase, then two possible signalling pathways can occur. Hor directly acts on *evf* promoting its expression, or Hor promotes the expression of a second regulator, *evr*, which consequently regulates transcription of *evf*.

The rarity of natural bacterial pathogens in *Drosophila* [29,64] makes the relationship between *Pectobacterium* and this insect a very interesting case to study. Being non-lethal, this interaction allows us to study both the host and the microbial side on a dynamic system and determine the influence of bacterial cell to cell communication systems in this host-microbe interaction.

In this work we have used the *P. carotovorum-Drosophila* model to study the role of QS in this interaction *in vivo*. We have studied the effect of Ecc15 on host physiology and identified a new phenotype for Evf which provides new tools to study its function. We also investigated the regulation of *evf* expression by QS and by its known and putative regulators. Ultimately, we studied the function of Evf in the context of this host-microbe interaction and the specific mechanism through which this protein changes the normal gut physiology of the host.

2. Materials and methods

2.1 Bacterial strains and growth conditions

All strains, plasmids and primers used in this study are listed in **Table 1** and **Table 2**, respectively. *P. carotovorum* strains used are derived from wild-type (wt) strain Ecc15[15] and were routinely grown at 30°C with aeration in Luria-Bertani (LB) broth. Antibiotics were used at the following concentrations, except otherwise specified: ampicillin (amp) 25 mgL^{-1}; kanamycin (kan) 50 mgL^{-1}; spectinomycin (spec) 50 mgL^{-1}; chloramphenicol (cm) 10 mgL^{-1}. Optical density (OD600 nm) was determined by measuring absorbance at 600 nm in a spectrophotometer (Spectronic, Thermo Scientific).

Table 1 – Strains and plasmids used in this study.

Strain	Relevant Genotype	Source
P. carotovorum		
Ecc15	Wild-type (wt)	[15]
Ecc15 *evf*::Tn10	*evf*::Tn10(*kan*)	[60]
Ecc15 pOM1-Evf	wt carrying pOM1-Evf	[60]
FDV67	wt carrying pLIPS	This study
FDV96	*ΔexpI::cm*	This study
FDV82	Δ*gacA::kan*	This study
FDV57	*Δhor::kan*	This study
FDV85	Δ*evr::kan*	This study
FDV74	wt carrying pFDV74	This study
FDV92	FDV96 carrying pFDV74	This study
FDV87	FDV82 carrying pFDV74	This study
FDV89	FDV57 carrying pFDV74	This study
FDV94	FDV85 carrying pFDV74	This study
FDV75	wt carrying pFDV75	This study
FDV93	FDV96 carrying pFDV75	This study
FDV88	FDV82 carrying pFDV75	This study
FDV90	FDV57 carrying pFDV75	This study
FDV95	FDV85 carrying pFDV75	This study
Plasmids	**Relevant genotype**	**Source**
pOM1	Cloning vector, *spec*^{r}	[65]
pUC18	Cloning vector, *amp*^{r}	[66]
pLIPS	pOM1 vector containing λ red recombinase system, *spec*^{r}	This study
pFDV74	pOM1 vector containing Promoter *hor-gfp*, *spec*^{r}	This study
pFDV75	pOM1 vector containing Promoter *evr-gfp*, *spec*^{r}	This study

Table 2 – Primers used in this study to construct the GFP reporter fusions, engineer the homologous recombination fragments and for confirmation of deletion mutants.

Primers Name	Sequence
1003-PevR_FW (XbaI)	CCTTACTCTAGAAATGACATGTTTTTTCCT
1004-PevR_RV (SphI)	CGAGCTGCATGCAAAATCATTCAACCCAATGGATA
1005-Phor_FW (XbaI)	CCTTACTCTAGATCAATAAATAGAGTTGTCGCGGG
1006-Phor_RV (SphI)	CGAGCTGCATGCCACCTCTCCTTATTGTTAGCACG
1108-Redsystem(pKD46)FWsphI	CCTTACGCATGCCATCGATTTATTATGACAA
1109-Redsystem(pKD46)RVXbaI	CGAGCTTCTAGATACCCATGGATTCTTCGTCT
1127-500Hor500RVSalI	CGAGCTGTCGACGCTAAACAGGTGCAGACCGT
1128-500Hor500FWSalI	CCTTACGTCGACTCAATAAATAGAGTTGTCGCGGG
1129-500Evr500RvSalI	CGAGCTGTCGACCCAGCGCGGCATAACTTTCG
1130-500gacA500FwSalI	CCTTACGTCGACTATGATGTTCACTATGGACG
1131-500gacA500RvSalI	CGAGCTGTCGACGATATTGCAGGCAGGGGCG
1087-HorDelRVXhoI	CGAGCTCTCGAGCACCTCTCCTTATTGTTAGC
1088-HorDelFWXhoI	CCTTACCTCGAGCTAAATTTGGGTTACGCAGA
1090-EvrDelRVXhoI	CGAGCTCTCGAGAGTGCAATCTCCATTTACCT
1091-EvrDelFWXhoI	CCTTACCTCGAGGTTTTATCTCATTATATTAG
1132-DelGacARvXhoI	CGAGCTCTCGAGGAATAATTCTCCAAAAAAGGG
1133-DelGacAFwXhoI	CCTTACCTCGAGGAGTTTCGATGCGTCGGCAT
1134-DelExpIFwXhoI	CCTTACCTCGACTTGCACAGGCTTGATGAGCTGTA
1135-DelExpIRvXhoI	CGAGCTCTCGAGCCTCCATTGAAAAGTTAATAC
1136-500ExpI500FwSalI	CCTTACGTCGACGAATACCGTGTCTGACAACC
1137-500ExpI500RvSalI	CGAGCTGTCGACATCGCCTTTCTCTTGGGAGA
1186-HorDelFw	AATCGTCAGTTATTACAATGGT
1187-HorDelRv	TATGATGAAGCGTTTGCTTGTG
1188-EvrDelFw	TTGATGGTGTAACGTGATCGT
1189-EvrDelRv	GGGCCTAATAGCCCAGTTGTTT
1190-ExpIDelFw	TCAGGCGCTGATGCTGCGTGAT
1191-ExpIDelRv	TCCAGTTATCCCGATGAATGGG
1192-GacADelFw	GGGCGTTACCGCTGACGCGACA
1193-GacADelRV	CAGGCGAACATAGTCAACCTGC
0665-GFP_RV	CCTTACGGATCCTCAGTTGTACAGTTCATCCATGCCA
0576-GFP_FW	CCTTACGCATGCATGGCTAGCAAAGGAGAAGAACTCT
0531_pOM1seq_R	ATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTC
0752-pOM1_seq2_F	CGCCCAATACGCAAACCGCCTCTCCCCGCGCGT

2.2 Genetic and molecular techniques

Unless otherwise mentioned, all PCR reactions were performed using Dream Taq Polymerase (Fermentas). Digestions were done using Fast Digest Enzymes (Fermentas) according to manufactures instructions and ligations performed with T4 DNA ligase (New England Biolabs)(See appendices). Electrocompetent cells were prepared using the glycerol method (See appendices) and a 2.2V shock was applied to favour DNA entry in the competent cells. DNA concentrations were measured using NanoDrop.

2.3 Construction of Ecc15 mutants by homologous recombination

The plasmid pLIPS was constructed by introducing the arabinose inducible λ Red recombinase system[67] into the pOM1 vector. The λ Red recombinase system (composed by the genes *araC*, *gam*, *bet*, *exo* and *af60A* total of 3421 bp) was amplified by PCR from the vector pKD46[68] using the Bio-x-act proof reading enzyme and the primers P1108-λRedsystem(pKD46)FW (SphI) and P1109-λRedsystem(pKD46)RV (XbaI). The fragment was digested with SphI/XbaI and ligated to the SphI/XbaI digested pOM1.

Mutants were constructed by chromosomal gene replacement with an antibiotic marker using the λ Red recombinase system. The DNA region of the gene to be replaced, including approximately 500 bp upstream and 500 bp downstream of the gene, was amplified with Bio-x-act and cloned into pUC18. The 500bp-*expI*-500bp, 500bp-*gacA*-500bp, 500bp-*hor*-500bp and 500bp-*evr*-500bp fragments were cloned into pUC18 using SalI. These constructs, containing the gene to be deleted and its flanking regions, were divergently amplified by PCR using the primers containing XhoI (1087-1135) from table 2 to introduce a XhoI restriction site in the 5' and 3' regions of the gene to be deleted. The antibiotic resistance genes *cm* or *kan* were amplified with primers containing the XhoI site, from pKD3[68] and pKD4[68] respectively, digested with XhoI and ligated to the XhoI digested PCR fragments. The final constructs contained the antibiotic marker flanked by the upstream and downstream regions of the gene to be replaced. The 500bp-antibiotic-500bp fragment was amplified by PCR using Bio-x-act and before electroporation a clean-up was performed using a PCR purification kit (Quiagen). The fragment was eluted in 50 µl of miliQ water and approximately 3000 ng of DNA was electroporated in 50 µl of Ecc15 electrocompetent cells expressing the λ Red recombinase system from pLIPS, to allow recombination. Recombinants were selected in LB plates with the respective antibiotic (kan 50 or cm 10). In order to lose the plasmid pLIPS after recombination, chosen recombinants were streaked in LB plates. The plates were then incubated at 30^{o}C or 37^{o}C (to induce temperature stress and increase the probability to lose the plasmid) and random colonies streaked in LB spec plates to check for plasmid loss.

2.4 Construction of the promoter gfp reporter fusions

The pFDV74 and pFDV75 vectors were constructed by introducing the promoter region of the genes *hor* and *evr* into pOM1 vector[65]. *hor* and *evr* promoter regions located 500 bp upstream of the genes starting codon were amplified by PCR from genomic DNA using Bio-x-act proof reading enzyme and primers P1005-Phor_FW (XbaI) and P1006-Phor_RV (SphI) for *hor* and primers P1003-Pevr_FW (XbaI) and P1004-Pevr_RV (SphI) for *evr*.

Both fragments were digested with XbaI/SphI and ligated to the XbaI/SphI digested pOM1. A 792 bp *gfp* promoterless fragment was amplified from pCMW1 and cloned into pOM1 adjacent to the promoter region fragment using the primers 0576-GFP_FW (SphI) and 0665-GFP_RV (BamHI).

2.5 Time-course gfp analysis of the promoter gfp expression

P. carotovorum strains containing the reporter plasmids pFDV74 and pFDV75 were grown overnight in LB+Spec and inoculated into fresh medium at a starting OD600nm of 0.05. At the indicated time points, aliquots were collected to evaluate growth and to assess *hor or evr* expression**.** For the analysis of *hor* or *evr* expression, aliquots of the cultures were diluted 1:100 into Phosphate Buffered Solution (PBS) and P*hor*-GFP or P*evr*-GFP expression was measured by flow cytometry (LSR Fortessa, BD) and analysed with Flowing Software v2.5.0. A minimum of 10,000 cells were acquired per sample and *hor* or *evr* expression was recorded as the median of GFP expression. All assays are reported as the mean of the median GFP expression from at least three biological replicates and error bars represent the standard deviation.

2.6 *P. carotovorum* virulence assay in potatoes

Virulence was analysed using a modified protocol to assess maceration of potato tubers[69]. Potatoes were washed and surface sterilized by soaking for 10 min in 10% bleach followed by 10 min in 70% EtOH. Overnight cultures were washed twice and diluted to an OD600nm of 0.05 in PBS and 30 µL were inoculated in the previously punctured potatoes. Potato tubers were incubated at 28^{o}C in a humid environment and at 48 h of incubation, potatoes were sliced and macerated tissue was collected and weighed. All assays are reported as the mean of the maceration from at least five biological replicates and error bars represent the standard error of the mean.

2.7 *Drosophila* oral bacterial infection and development assay

Transgenic Oregon^{r} larvae carrying a *gfp* gene tagged to *diptericin* promoter region[70] in both arms of the third chromosome were used in all assays. Egg laying was performed in cages containing 40 adult flies at a ratio of 3 females to 1 male. The flies were incubated for 4 to 6 hours at 25^{o}C in the presence of standard corn meal fly medium. After this period, the eggs were removed and incubated at 25^{o}C for 70 hours to obtain L3-stage larvae.

For the bacterial infections, approximately 30 third-instar larvae were placed in a 2 ml Eppendorf containing 200 µl of concentrated bacteria pellet (OD600 = 200) from an overnight culture and 400 µl of standard corn meal fly medium. Larva, bacteria and food were

thoroughly mixed in the Eppendorf. The Eppendorf was then closed with a foam plug and incubated at room temperature for 30 min. The mix was then transferred to a 25 ml plastic tube containing 7.5 ml of standard corn-meal fly medium and incubated at 28^{o}C. The development of the larvae post-infection, comprising the larva to pupa and pupa to fly transition, was then followed for 8 days. The frequency of larvae that proceeded to the pupae stage was assessed by counting the number of pupae present inside the plastic tube. Similarly, the frequency of eclosed flies was determined based on the number of flies that emerged from the pupae. Unless otherwise mentioned, all assays are reported as the mean of frequency from at least five biological replicates and error bars represent the standard error of the mean.

2.8 Oral infection with Supernatants

For each strain, 100 ml of an overnight culture were centrifuged at 4000 RPM for 20 min at room temperature, in an Eppendorf centrifuge model 5810 in order to pellet the bacteria. The supernatant was transferred to a new recipient and bacterial cells were discarded. Supernatants were then filtered through a 2 µm diameter pore filter (Pall Corporation) to remove remaining bacteria or cellular debris. 200 µl of the supernatant were then used to infect larvae following the same protocol described above in the ***Drosophila oral bacterial infection and development assay*** section.

2.9 Food dye quantification

To evaluate the amount of food ingested by the larvae post-treatment, pigment quantification in larvae fed with blue dyed fly medium was performed. Larvae were treated according to the protocol described previously for the infection with some changes. After the 30 min infection period larvae, bacterial pellet and food were transferred to a new plastic tube containing standard corn meal fly medium to which was added 30,5 µl of a blue food dye (Rayner's). At the indicated time points, larvae were collected from the tubes using a 15% sucrose solution and washed in a petri dish containing PBS 1x. 30 larvae were randomly collected to an Eppendorf and homogenized with a blender in 200 µl of 80% methanol. The suspension was then centrifuged for 10 min to deposit larvae residue. 100 µl of the mixture methanol /blue dye were recovered and measured at 625 nm using the multiskanGO (Thermo scientific). All assays are reported as the mean of absorbance (625 nm) from at least five biological replicates and error bars represent the standard error of the mean.

2.10 Statistical analysis

The data presented here were analyzed using Graphpad Prism6 software and program R version 3.0.2. The Mann-Whitney test was performed to evaluate significance and P-values were adjusted using the Holm-Bonferroni correction for multiple comparisons. An adjusted P-value <0.05 was used as the cut-off for statistical significance. ns Not significant; *P-value <0.05 and **P-value <0.01.

3. Results

3.1 Ecc15 infection causes a development delay in *D. melanogaster*

It was recently shown that single acquisition of the gene *evf* provided *P. carotovorum* strain Ecc15 the ability to persist in the gut of *D. melanogaster*[60,71]. This persistence triggered the activation of the immune system, resulting in a state of transient infection. Though the host immune response against *P. carotovorum* was investigated, the impact of the *evf*-dependent infection in host development was never assessed. In order to understand the effect of this infection in host development we monitored the development of larvae over time, by counting the numbers of larvae which progressed to the pupation stage after infection with Ecc15 (Figure 4). L3 stage larvae were fed with a mixture of standard corn meal fly food and a bacterial pellet or, as control, only with standard fly food.

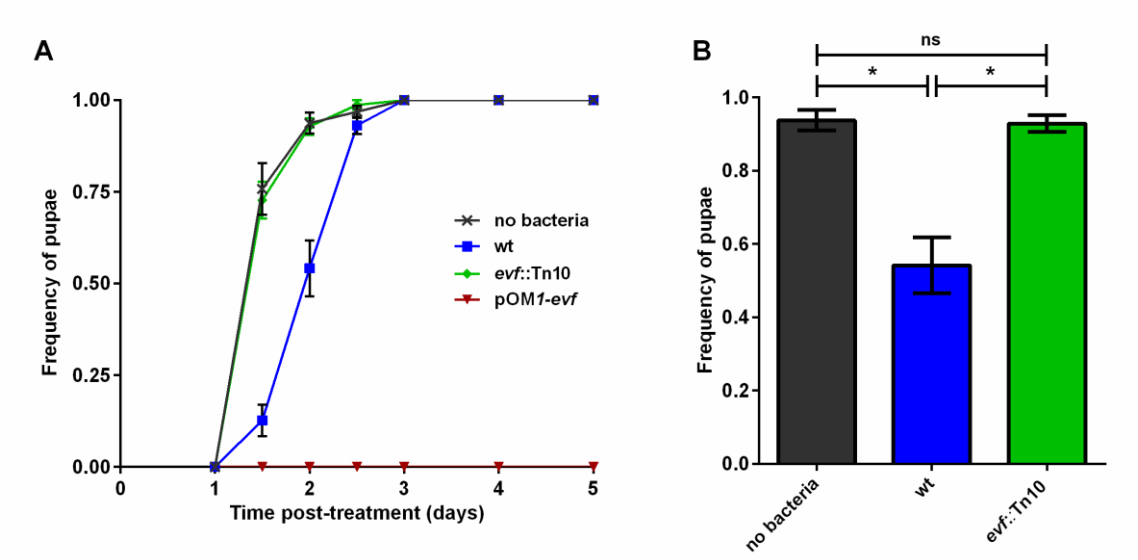

Figure 4 – Effect of Ecc15 on *D. melanogaster* larvae development. Larvae pupation frequency was evaluated by counting the number of larvae (30±5), reaching pupae stage after infection with Ecc15 (wt) (blue), *evf* mutant (green) bacteria and Ecc15 pOM1-*evf* (strain overexpressing *evf*) (red) in each independent replicate (n=5). As control larvae were fed with standard fly food without bacteria (dark grey). (A) Time-course frequency of pupation after infection. (B) Represents the average pupation frequency of each treatment at day 2. Error bars represent the standard error of the mean, * stands for Pvalue<0.05 and ns for Not significant.

The results showed that the majority of the control larvae reached the pupae stage during the first two days after infection (Figure 4A, dark grey crosses). In opposition, larvae fed with wt Ecc15 displayed a development impairment of approximately one day when compared to the control (Figure 4A, blue squares and dark grey crosses), only reaching 50% of pupae in two days (Figure 4B, blue bar and dark grey bar). Importantly, larvae fed with an *evf* mutant strain of *P. carotovorum* had no delay in development (Figure 4B, green bar) displaying a temporal

dynamic similar to the control larvae not exposed to bacteria (Figure 4A, green diamonds and dark grey crosses). Moreover, larvae infected with Ecc15 overexpressing *evf* died without reaching the pupa stage (Figure 4A, red inverted triangles).

These results demonstrated that infection of *D. melanogaster* with Ecc15 had a significant impact in larvae development. Moreover, the observed development delay could be specifically attributed to *evf,* since this effect was absent in infections with mutants lacking this gene and led to larvae lethality when it was overexpressed. However, the mechanisms behind this phenotype are not clear, highlighting the importance of studying the effect of *evf* in the context of infection and development of larvae.

3.2 Ecc15 changes the feeding behaviour of *D. melanogaster* larvae

The control of body size is essential for the development of both insects and mammals. Several studies performed in insects, and in particular in *D. melanogaster*, showed that this parameter is regulated by molecular, environmental and physiological mechanisms, such as critical weight[48,49]. The critical weight is the development switch where no more growth is needed to enter the pupation stage[55]. Several environmental factors such as temperature, larval density or nutritional quality are important to the regulation of body size and consequently have an impact on the critical weight. Our previous results showed that larvae fed with wt Ecc15 had a development delay, which could be related with a problem in attaining the critical weight necessary to commit to pupae stage. Since both temperature and population size were well controlled in our experiments, a likely cause to this delay was a nutritional problem. Supporting this hypothesis, we noticed that larvae infected with wt bacteria were thinner than both control larvae and larvae infected with an *evf* mutant, suggesting an altered nutrition in wt-infected larvae. Therefore, in order to test our hypothesis, we monitored the feeding behaviour of infected larvae, by staining the standard fly food with a blue dye (Rayman's) and quantifying the amount of blue pigment internalized by the larvae at the indicated time points (Figure 5). Strikingly, 3 hours after treatment, larvae infected with wt bacteria (left blue bar) displayed a reduced amount of internalized pigment, suggesting that they were indeed consuming little or no food. This contrasts to what is observed for the control or *evf*-infected larvae (left dark grey and green bars, respectively), where the amount of pigment detected indicates food ingestion.

At 22 hours post-treatment we could observe the presence of the pigment in wt-infected larvae (right blue bar), which shows that by this time they had resumed food consumption. This observation suggests that with time the infection with wt bacteria preventing food uptake was eliminated, allowing the larvae to resume food consumption until

reaching the critical weight development checkpoint needed to enter the pupation stage. Interestingly, even after 22 hours post infection we could still observe a difference in the amount of food ingested by the wt-infected larvae, showing the impact of the transient *P. carotovorum* infection for the larvae development (Figure 5, right blue bar with green and dark grey bars).

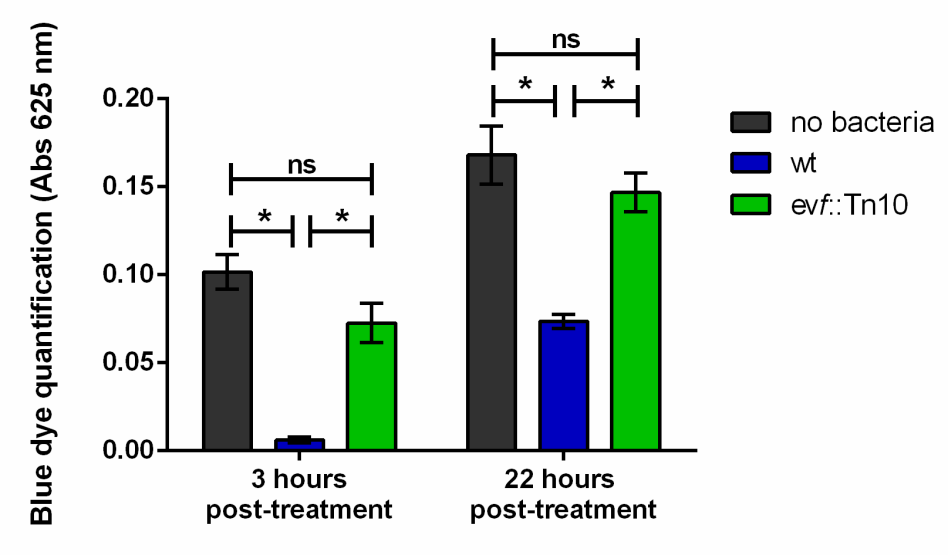

Figure 5 – Estimation of food uptake by *D. melanogaster* larvae after infection with Ecc15. Food uptake was inferred by quantifying the amount of blue pigment (Abs 625 nm) internalized by non-infected control larvae (dark grey bars), Ecc15 wt-infected (blue bars) or *evf*::Tn10-infected (green bars). For each treatment a pool of 30 larvae was collected (n=5). Error bars represent the standard error of the mean, * stands for Pvalue<0.05 and ns to Not significant.

Our results showed a transient nutritional impairment in *D. melanogaster* larvae mediated by *evf*. Bacteria lacking this gene were unable to disrupt the normal larval feeding behaviour, contrary to what was observed in the first hours in wt-infected larvae. This in turn suggests that Evf has a temporary toxic effect on larvae, impacting the progression of the life cycle of the fly.

3.3 Evf function at the host level

In 2007 Muniz *et al* hypothesized that *evf* was involved in blocking the peristaltic movements occurring in the larvae gut. Presumably, Evf would interfere with the natural movements that eliminate bacteria from the intestinal apparatus, allowing the increase of their titters inside the gut. However, the specific mechanism of action of Evf remains unknown. In order to understand if Evf acts as an extracellular toxin secreted by Ecc15, we compared the development of *D. melanogaster* larvae exposed to bacterial cell-free supernatants or corresponding bacterial cultures from wt, *evf* mutant and Ecc15 overexpressing *evf* (Figure 6). To do that, we collected bacteria cell-free supernatants from a filtered overnight culture of the respective strain. The supernatant was then given to larvae following the same protocol described before (see materials and methods).

As we previously showed, larvae infected with wt (Figure 6, blue squares) showed a development delay, since at day 2 only 22% had reached pupae stage, contrasting with the 90% observed in the control larvae (Figure 6, dark grey crosses). Also in agreement with previous experiments, 80% of *evf* mutant-infected larvae (Figure 6, green diamonds) reached the pupae stage by day 2, similarly to the control (Figure 6, dark grey crosses). As we expected, larvae to which supernatant from the *evf* mutant (Figure 6, empty purple diamonds) was given showed no development delay. However, the development delay was also absent in larvae fed with wt supernatant (Figure 6, empty orange squares), in opposition to the effect observed in wt-infected larvae (Figure 6, blue squares). This result indicates that the Evf-dependent effect is not caused by extracellular secretion. Supporting this hypothesis, larvae infected with the supernatant of an *evf*-overexpressing strain of Ecc15 (Figure 6, red hexagons) also exhibited no delay in development, despite the lethal effect observed in larvae infected with Ecc15 pOM1-*evf* (Figure 4, red inverted triangles).

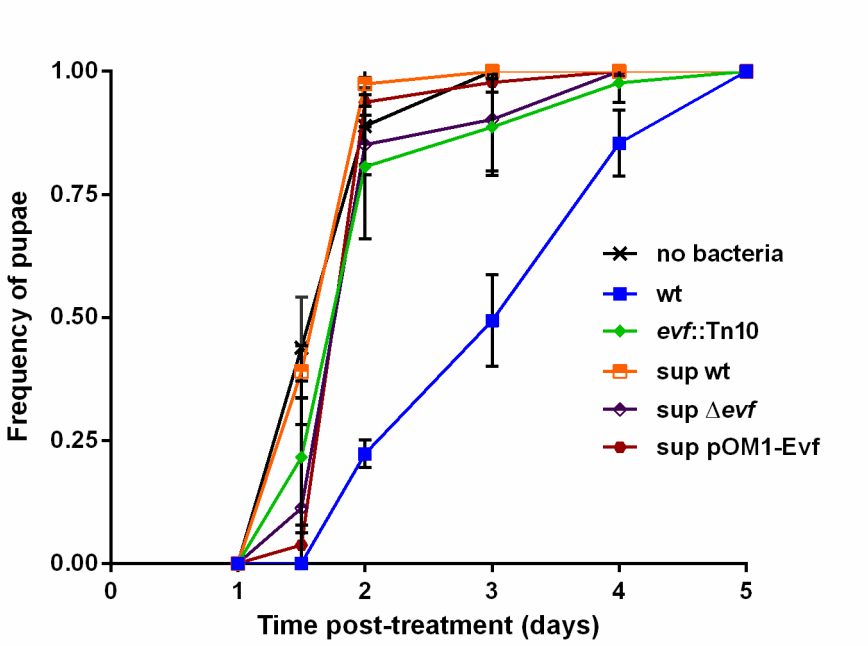

Figure 6 – Effect of bacterial supernatants in the development of *D. melanogaster* larvae. Larvae pupation frequency was evaluated by counting the number of larvae (30±6) reaching pupae stage after infection with Ecc15 (wt) (blue squares) or *evf* mutant (green diamonds) bacteria or supernatants from ON cultures of Ecc15 (wt) (orange half full squares), *evf* mutant (purple half full triangles) and *evf*-overexpressing strain pOM1-*evf* (red hexagons). As control larvae were fed with standard fly food without bacteria (dark grey crosses). Error bars represent the standard deviation of 3 independent replicates.

To further elucidate the mechanism responsible for the larvae development delay we evaluated the specific effect of the Evf protein on larval development. To address this question we administered purified Evf protein to *D. melanogaster* larvae. Since the amount of Evf protein produced by Ecc15 was never determined, we assumed that it could be comparable to the concentration of toxins produced by entomopathogenic bacteria, such as *Photorhabdus luminescens*. This bacterium produces a battery of toxins with insecticidal activity that are effective when administrated both orally and injected directly into the hemolymph[72]. These toxins were found to be active in small concentrations specially when injected[73]. In the literature, to test oral activity of *Photorhabdus* toxins, concentrations used ranged from 0.2 to 20 µg/ml[73]. Based on these findings, we applied two different concentrations of purified Evf protein (370 µg/ml and 37 µg/ml) to the larvae following the same protocol described before for the infection experiments.

As shown above, bacteria-infected larvae displayed the typical development dynamics: delay in larvae infected with wt and normal development in *evf* mutant-infected larvae, similarly to the control (Figure 7). In respect to the larvae exposed to Evf protein, the lower protein concentration (37 µg/ml) used did not affect the larval development (dark green

diamonds, Figure 7A and bar, Figure 7B), which resembled that of control larvae. With higher protein concentrations (370 µg/ml) we observed a slight difference in larvae development compared to the control larvae (purple inverted triangles, Figure 7A and bar Figure 7B). However, this difference is too small, considering the amount of protein applied.

Due to the small number of replicates we could not test our results for statistical significance. However, considering the strength of our phenotype and the amounts of protein used the small difference between the two concentrations of protein does not seem to be relevant, rejecting the hypothesis that Evf could act extracellularly.

Together these results suggest that the product of the *evf* gene does not act as a secreted protein, since neither the cell-free bacterial supernatant nor the purified Evf protein had a dramatic effect on *D. melanogaster* larval development. Therefore, this data seems to indicate that the presence of the bacteria is essential to exert the *evf*-dependent toxic effect on the larva that leads to the observed developmental delay.

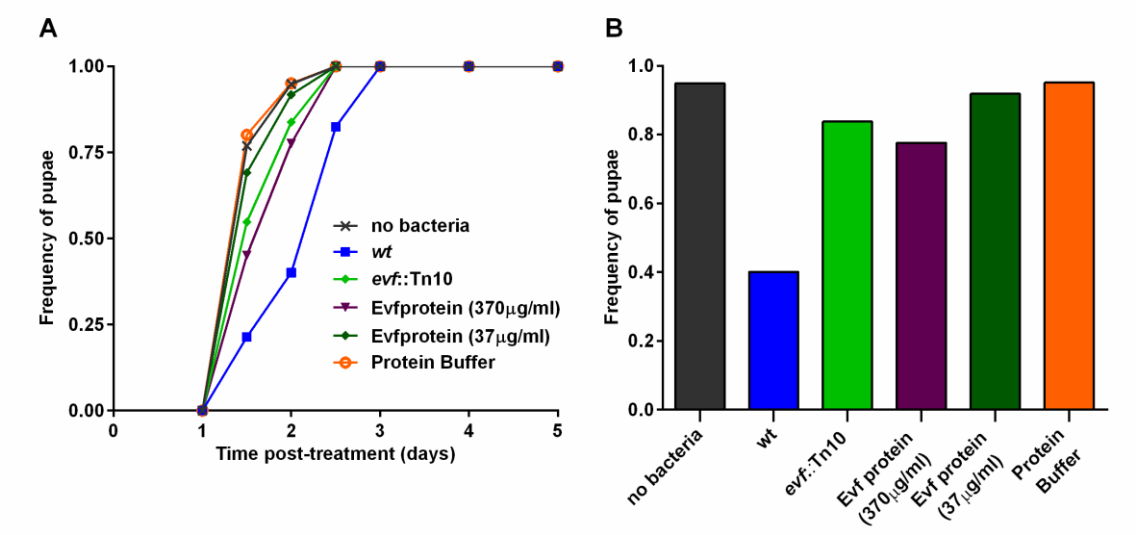

Figure 7 – Effect of the purified Evf protein on *D. melanogaster* larvae development. Larvae pupation frequency was evaluated by counting the number of larvae, reaching pupae stage after infection with wt (blue squares and bar), *evf* mutant (green diamonds and bar) bacteria and 2 different concentrations of purified Evf protein (purple inverted triangles and bar; dark green diamonds and bar) in each independent replicate (n=2). As control larvae were fed with standard fly food without bacteria (dark grey crosses and bar). (A) Time-course frequency of pupation after infection. (B) Represents the average pupation frequency of each treatment at day 2.

3.4 Network controlling Evf production

Having established an assay to study the Ecc15-*Drosophila* interaction and after showing the strong dependence on *evf* in this interaction we next studied the genes involved regulating Evf production and the possible role of QS control in this process. Little was known about the regulation of *evf* in these bacteria. In fact, until now, no genetic tools were available to construct gene deletions in this strain of *P. carotovorum,* and the only mutants available

were mutants selected from a library of random transposon mutants[60,74]. Thus to study the regulation of *evf* we developed and optimized a method to construct gene deletion mutants in Ecc15 (see detailed information in Materials and methods section). A set of deletion mutants in both QS (*expI* and *gacA*) regulatory pathways and the genes reported to be involved in regulating Evf (*hor* and *evr*) were constructed. *expI* codes for the AHLs synthase whereas *gacA* encodes the response regulator of the two component system GacS/GacA. *hor* codifies for a protein from the slyA family and is the only regulator in which the role on *evf* regulation has been described. *evr* is described as a regulator of *evf* (http://www.ncbi.nlm.nih.gov/nuccore/AY167733.1) but its role was never assessed. The mutants were constructed by homologous recombination using an adaptation of the Red-swap method originally described by Datsenko and Wanner[68] in *Escherichia coli*. In this method the plasmid pKD46 containing an arabinose inducible lambda phage recombinase system is usually used to enable recombination events from linear DNA fragments, which would otherwise be targeted for degradation. However, this plasmid could not be used in Ecc15. To solve this limitation we cloned the lambda red recombinase system into pOM1, a plasmid commonly used in Ecc15, creating pLIPS, which was successfully introduced in *Pectobacterium* Ecc15. The Ecc15 strain (**FDV67**) containing the pLIPS plasmid was then used as the parent strain to construct all the desired mutants. The *expI*, *gacA*, *hor* and *evr* Ecc15 mutants were constructed by recombining a linear DNA fragment containing an antibiotic cassette (*kan* or *cm*) flanked by, approximately, the -500 and the +500 bp of the gene to be deleted in the chromosome. These large homology regions are important to increase recombination efficiency. Putative recombinants were confirmed by PCR using genomic DNA as template, since attempts to perform colony PCR in Ecc15 were unsuccessful. The deletions were confirmed by PCR using primers flanking the -600 and +600 region of the target gene and outside the recombination region (table 2 materials and methods), in order to guaranty that the recombination had occurred at the proper place and to exclude non homologous recombination events. Products of the PCR reactions and expected band sizes are presented in Figure 8 and table 3 respectively.

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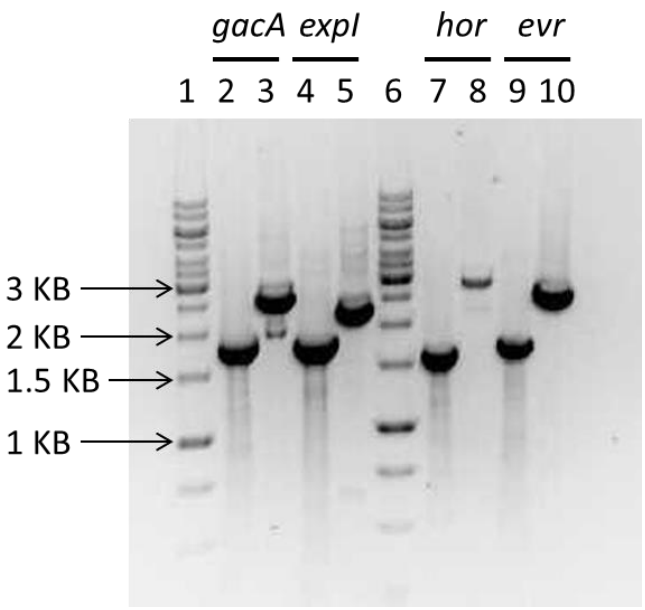

Figure 8 – Confirmation by PCR of the construction of Ecc15 mutants using a Red-swap approach. Colonies were selected in the respective medium (LB+ *kan* or *cm*), genomic DNA was extracted and gene deletion was checked by PCR using primers that flanked the deleted region: 1 and 6- 1 KB ladder (Fermentas), 2- wt *gacA* (1857 bp), 3- Δ*gacA::kan* (2702 bp), 4- wt expI (1851 bp), 5- Δ*expI*::*cm* (2235 bp), 7- wt *hor* (1638 bp), 8- Δ*hor::kan* (2702 bp), 9- wt *evr* (1746 bp), 10- Δ*evr::kan* (2702 bp).

Table 3 – Expected band sizes for *P. carotovorum* strain Ecc15 mutants in QS systems (*expI* and *gacA*) and *evf* regulators (*hor* and *evr*).

		Expected band size (bp)	
Gene	**Primers**	**Mutant (gene::antibiotic cassette)**	**Wild-type**
expI	1190; 1191	2235	1851
gacA	1192; 1193	2702	1857
hor	1186; 1187	2702	1638
evr	1187; 1189	2702	1746

As shown in Figure 8, the expected band size for each mutant was obtained and the difference to the wt was clear. Moreover the recombination occurred at the right sites since our primers were designed to anneal to the sequences flanking the region to be deleted.

3.5 Phenotypes of Ecc15 mutants in plant virulence

Since Ecc15 belongs to the *P. carotovorum* and many strains from this species are enteric phytopathogens that infect several vegetables and regulate their virulence factors via QS, we investigated the virulence of our strain Ecc15 in vegetable hosts and study the effect of the mutants constructed here. We tested our wt and *evf* mutant strains, as well as the newly constructed mutants on cell-to-cell communication systems (*expI* and *gacA*) and *evf* known

and putative regulators (*hor* and *evr* respectively) for their ability to degrade plant components. We used a modified protocol to measure maceration of potato tubers[69]. Potatoes were punctuated, inoculated and maceration was accessed 48 hours after infection. As shown in Figure 9, after 48 hours potatoes infected with the wt strain displayed considerable levels of maceration (blue circles). In contrast, Ecc15 mutants in the QS systems (Δ*expI* and Δ*gacA*) caused low levels of potato maceration (orange squares and green triangles, respectively) when compared to the wt. Regarding the *evf* regulators, the *hor* mutant (red inverted triangles) was also impaired in virulence when compared to wt (blue circles). Because the level of maceration obtained in potatoes infected with the *evf* mutant (red open circles) were comparable to those observed in wt-infected potatoes (blue circles), this suggests a possible role of Hor in regulating other functions related to virulence beyond *evf* regulation

Interestingly, potatoes infected with Δ*evr* displayed a partial phenotype (yellow diamonds), in which the levels of maceration were approximately 2/3 of that observed in wt-infected potatoes (blue circles). This result indicates that *evr* can play a role in virulence expression but not as important as the cell-to-cell communication systems. These results demonstrated that Ecc15, like other *P. carotovorum* strains, regulates the factors required for maceration of tissue by QS and showed the importance of QS to virulence expression in this bacterium. Additionally, these results also showed that *evf* is not important for potato infections. This observation contrasts to what was observed in larvae infections, where the *evf* was essential for causing the deleterious effect on the host. Taken together these results suggest a particular role of *evf* in the infection of larvae, emphasizing the specificity of this interaction and supporting the theory of single gene acquisition for new niche invasions.

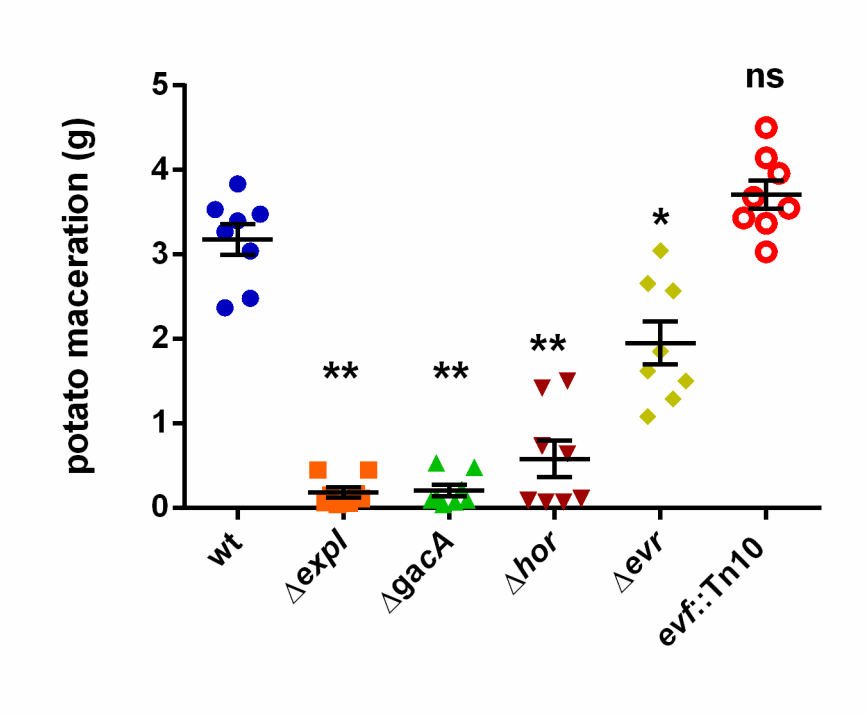

Figure 9 – Assessment of plant virulence in Ecc15 mutants. Production of PCWDE was assessed by measuring the maceration of potatoes (n=8) infected with Ecc15 (wt) (blue circles), FDV96 (Δ*expI::cm)* (orange squares), FDV82 (Δ*gacA::kan)* (green triangles), FDV57 (Δ*hor::kan)* (red inverted triangles), FDV85 (Δ*evr::kan)* (yellow diamonds) and Ecc15 *evf::*Tn10 (red open circles) bacteria. Each potato was punctuated, infected with the respective strain and maceration measured after 48 hours. Error bars represent the standard error of the mean, * stands for Pvalue<0.05, ** stands for Pvalue<0.01 and ns for Not significant.

3.6 QS regulation of *evf* expression

It was hypothesized that single acquisition of the gene *evf* allowed Ecc15 to persist in the gut of *D. melanogaster* larvae[60]. This single event partially changed the life style of this *P. carotovorum* strain, allowing it to explore a new environment. Our results on the larvae development, feeding behaviour and virulence in potatoes support this theory. However, for gene transfers to be successful, the gene has to be integrated in the regulatory network available in the bacterium, resulting in a regulated gene expression. Until now, due to the lack of genetic tools, the network regulating *evf* expression could not be properly explored.

We hypothesized that *evf* expression is regulated by QS via RsmA/*rsmB*, similarly to what occurs for the production of PCWDEs. In order to address this question we started by investigating the expression of the genes described as regulators of *evf* (*hor* and *evr*). For this we constructed report fusions which contain the promoter sequence of the target gene (P*hor* and P*evr*) fused to a promoterless *gfp* sequence (P*hor*-gfp and P*evr*-gfp). These fragments were then cloned into pOM1 and the resulting plasmids (pFDV74 e pFDV75) transformed in our newly constructed mutants of Ecc15 (Δ*expI*, Δ*gacA*, Δ*hor* and Δ*evr*), as well as in the wt strain.

GFP expression of the promoter fusions per cell was then analysed in a time course experiment using flow cytometry (Figure 10 and 11).

Our results showed that the presence or absence of the AHL QS signal changed the levels of expression in both genes, which is a common observation in QS-regulated gene expression[19,75,76]. In the Δ*expI* mutant, which does not produce AHLs, *hor* expression (orange squares Figure 10A, and bar Figure 10B) was lower than the wt (blue circles Figure 10A, and bar Figure 10B), but increased to levels similar to the wt when grown in the presence of supplemented exogenous AHLs (orange striped bar, Figure 10B). Regarding *evr* expression, we observed similar levels of GFP in Δ*expI* (orange squares, Figure 11A and bar, Figure 11B) compared to wt (blue squares Figure 11A, and bar Figure 11B). However, supplementation of this mutant with exogenous AHLs (orange striped bar, Figure 11B) resulted in increased expression to levels higher than in the wt (blue bar, Figure 11B), a trend already observed when analysing *hor* expression. This indicates a possible regulation by AHLs and consequently QS. Comparing both promoters it seems that *hor* is more strongly regulated by AHLs than *evr*. Even though in both promoters expression is enhanced by exogenous AHLs, the difference between the wt and the Δ*expI* mutant without supplementation of AHLs is more pronounced in P*hor*-gfp reporter. Despite the results with AHLs QS system, expression of both *hor* and *evr* promoter regions in a Δ*gacA* mutant (green triangles, Figure 10A and 11A), the response regulator in the second QS system, was equal to wt (blue circles, Figure 10A and 11A). This indicates that GacSA QS system does not play a role in expression of both genes.

Regarding *evf* regulator mutants, we observed that expression of P*hor*-gfp in the Δ*hor* (red inverted triangles, Figure 10C) and Δ*evr* (yellow diamonds, Figure 10C) strains was not different from the wt (blue circles, Figure 10C). This indicates that either *evr* does not regulate *hor* expression or if they belong to the same regulation network then *evr* is located lower in the hierarchy. Likewise, when analysing P*evr*-gfp expression in Δ*hor* (red inverted triangles, Figure 11C) we detected levels of GFP expression similar to the wt (blue circles, Figure 11C), indicating that *hor* has no influence in *evr* expression. Together with the previous data, this result indicates that *hor* and *evr* do not belong to the same regulation network and implies that if both genes regulate *evf* expression, then at least two regulatory networks exist. Although the presented data seem to support this scenario, to fully clarify this point the analysis of *evf* expression over time is required (work in progress). In addition, and interestingly, we observed in Δ*evr* (yellow diamonds, Figure 11C) an increased GFP expression, which indicates an *evr* expression higher than in the wt (blue circles, Figure 11C). This suggests the existence of a negative feedback regulation loop where *evr* represses its own expression, contrary to what was previously described in other *Pectobacterium* spp[77].

Importantly, our results also showed that the profile of gene expression in *hor* and *evr* was different from a standard QS-regulated gene[19]. The peak of expression occurred at 4 hours of growth for both reporters (see wt, represented in blue in Figure 10 and 11), which corresponds to middle exponential phase (Figure 10D and 11D), followed by decrease in expression at high cell density. This observation contrasts to what is typically observed in genes regulated by QS, where increased gene expression in proportion to cell density is observed. After 4 hours the expression of both genes decreased, indicating that at stationary phase these genes are repressed by a putative unknown repressor.

Together our results highlighted part of the gene network supposedly regulating *evf* expression. We concluded that QS plays a role in the expression of both *hor* and *evr* (stronger in *hor*), since we observed a differential early response dependent on the presence of AHLs. However, contrasting to typical QS regulation, at stationary phase expression is repressed by an unknown mechanism. The GacSA system do not seem to be important in the expression of both *hor* and *evr* genes, at least in *in vitro* experiments. We also showed that *hor* and *evr* belong to two different regulatory networks and none of them influences the expression of the other. Additionally we hypothesized that in case that both genes regulate *evf* expression, then possibly two different networks exist, a hypothesis that can be tested by analysing the expression of *evf* promoter sequence (work in progress).

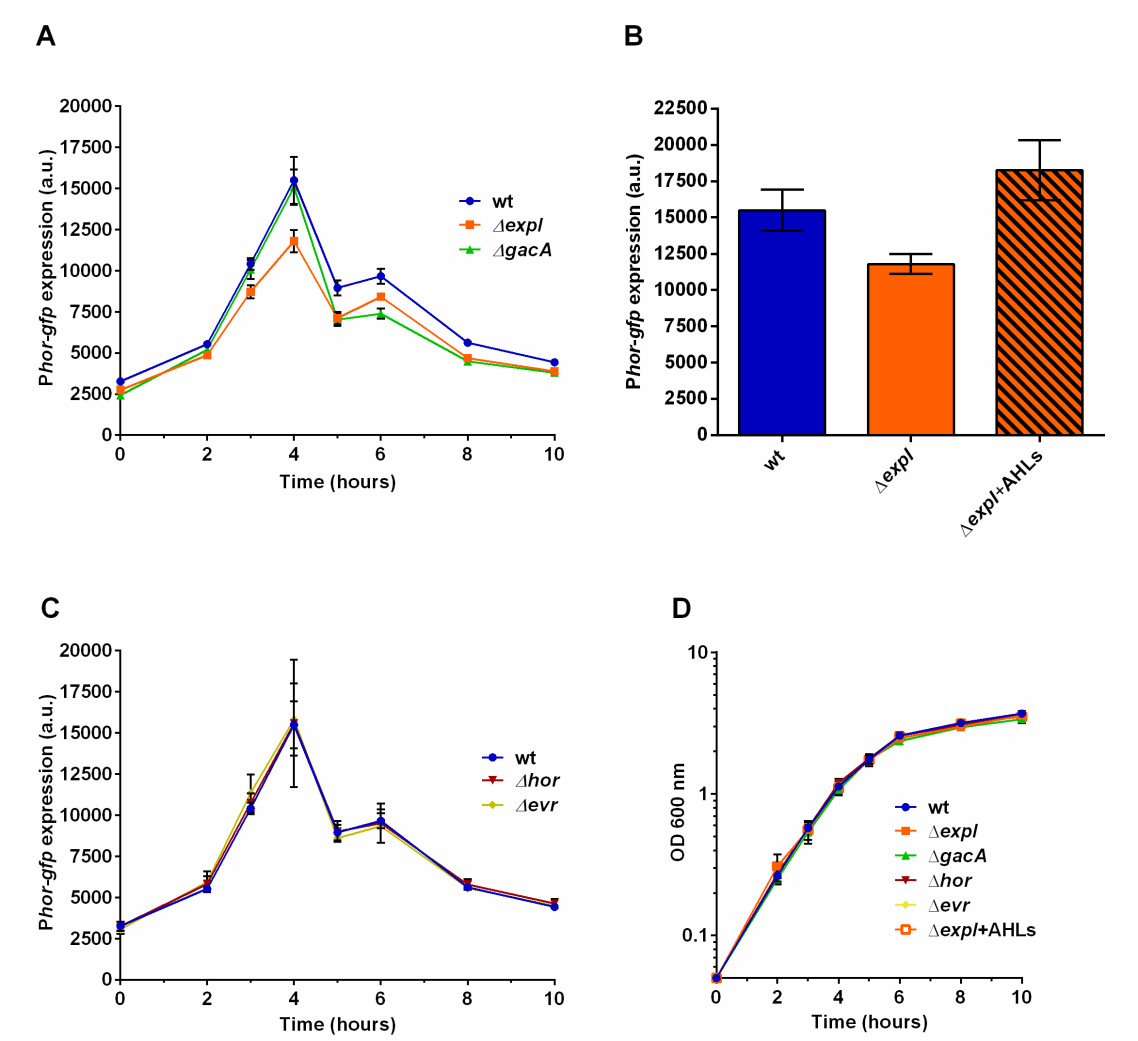

Figure 10 – Profile of *hor* expression in Ecc15 mutants. *hor* expression per cell was measured in LB + Spec (n=3) in a time course experiment (A, C) and after 4 hours of growth (B) using reporter strains containing the pFDV74 (Phor-GFP) plasmid. (A) Phor-GFP expression level per cell in Ecc15 (wt) (blue circles), FDV92 (Δ*expI::cm*)(orange squares) and FDV87 (Δ*gacA::kan*) (green triangles). (B) GFP expression of the Phor per cell at 4 hours of growth in Ecc15 (wt) (blue bar), FDV96 (Δ*expI*::*cm*) (orange bar) and FDV96 (Δ*expI::cm*) complemented with AHLs (orange striped bar). (C) Phor-GFP expression level per cell in wt (blue circles), FDV89 (Δ*hor::kan*) (red inverted triangles) and FDV94 (Δ*evr::kan*) (yellow diamonds). (D) Growth curve of Ecc15 strains carrying the reporter plasmid pFDV74. Error bars represent standard deviation of 3 independent replicates.

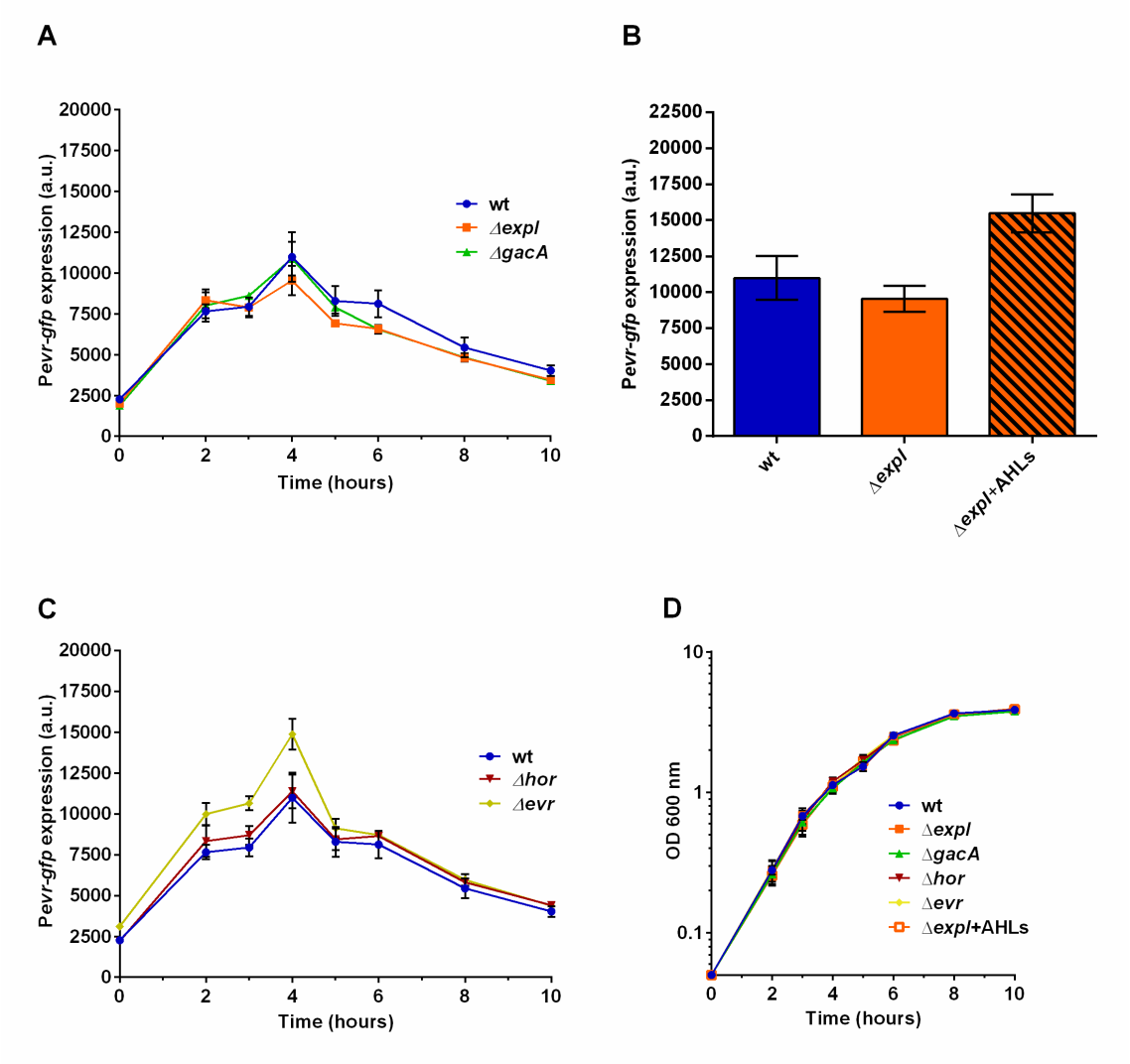

Figure 11 – Profile of *evr* expression per cell in Ecc15 mutants. *evr* expression per cell was measured in LB + Spec (n=3) in a time course experiment (A, C) and after 4 hours of growth (B) using reporter strains containing the pFDV75 (Pevr-GFP) plasmid. (A) Pevr-GFP expression level per cell in Ecc15 (wt) (blue circles), FDV93 (Δ*expI::cm*) (orange squares) and FDV88 (Δ*gacA::kan*) (green triangles). (B) GFP expression of the Pevr per cell at 4 hours of growth in Ecc15 (wt) (blue bar), FDV93 (Δ*expI::cm*) (orange bar) and FDV93 (Δ*expI::cm*) complemented with AHLs (orange striped bar). (C) Pevr-GFP expression level per cell in wt (blue circles), FDV90 (Δ*hor::kan*) (red inverted triangles) and FDV95 (Δ*evr::kan*) (yellow diamonds). (D) Growth curve of Ecc15 strains carrying the reporter plasmid pFDV75. Error bars represent standard deviation of 3 independent replicates.

3.6 Effect of QS and Evf regulator mutants in the development of *Drosophila* larvae

To further understand the effect of cell-to-cell communication systems and the role of the two regulators of *evf* in the context of the natural infection, we investigated the development of larvae infected with our newly constructed deletion mutants, *expI* and *gacA* for QS and for *evf* regulation, *hor* a known regulator and *evr* a putative regulator. Larvae were infected with the different mutants, including the *evf* transposon mutant previously described, as well as the wt, following the protocol described before for the infection experiments.

In this experiment, 83% of the control larvae reached the pupae stage during the first two days (Figure 12 and 13). As previously shown, larvae infected with wt Ecc15 displayed a development delay, with only 23% reaching the pupa stage two days after infection and larvae infected with *evf* mutant had a developmental dynamic similar to control larvae, with no delay in development (Figure 12, and 13). In respect to the QS influence in this system, the results showed that Δ*expI*-infected larvae (green triangles and bar, Figure 12A and B) showed no delay in development, similarly to what was observed in control larvae and the larvae infected with the *evf* mutant. This indicates that the AHLs QS system plays a role on the *evf*-mediated developmental delay or in the (unknown) effector mechanism of *evf*. Interestingly, larvae infected with Δ*gacA* (inverted red triangles, Figure 12A and B), the mutant in a second QS system, showed a significant development delay when compared to control, with only 53% of larvae reaching the pupae stage by day 2. However, it is a partial delay since its significant different from wt-infected larvae.

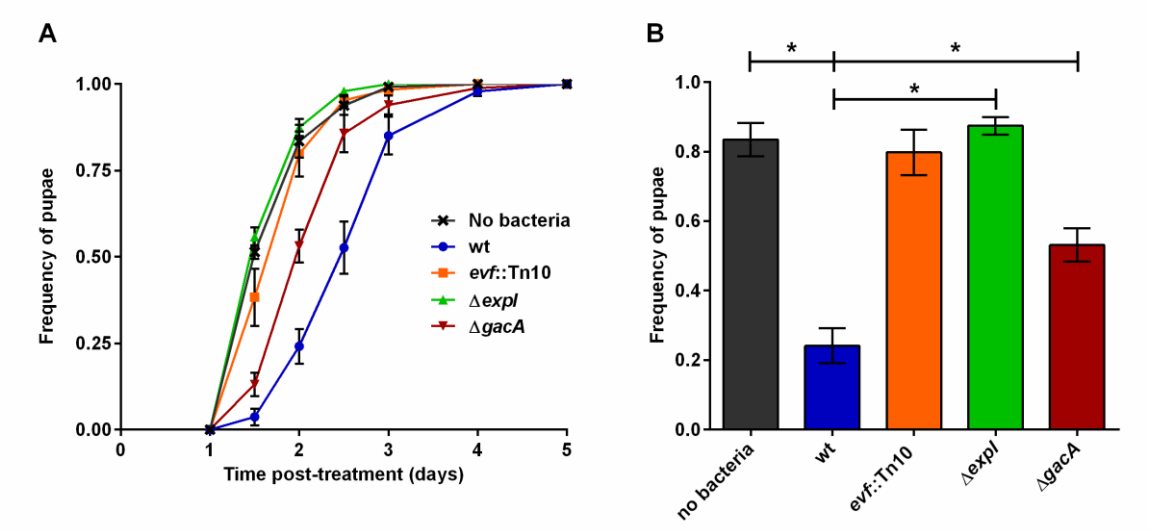

Figure 12 – Effect of QS mutants on the development of *D. melanogaster* larvae. Larvae pupation frequency was evaluated by counting the number of larvae (32±7), in each independent replicate (n=5), reaching pupae stage after infection. Larvae were fed with standard fly food without bacteria control, (dark grey crosses) or infected with Ecc15 (wt) (blue circles), *evf* mutant (orange squares), FDV96 (Δ*expI::cm*) (green triangles) and FDV82 (Δ*gacA::kan*) (red inverted triangles). (A) Time-course frequency of pupation after infection. (B) Represents the average pupation frequency of each treatment at day 2. Error bars represent the standard error of the mean, * stands for Pvalue<0.05 and ns for Not significant.

Regarding the *evf* regulators, we observed that larvae infected with Δ*hor* strain (yellow diamonds and bar, Figure 13A) had no development delay, resembling the *evf* mutant and control. This reinforces the role of *hor* as an important regulator of *evf* expression, as it was previously described[60]. Interestingly, larvae infected with Δ*evr* showed a developmental delay when compared to the control, with 42% of pupae by day 2 after infection. In fact, this delay is comparable to that observed when using the wt strain, since the statistical analysis revealed no significant difference between Δ*evr* and wt (blue and yellow bars, Figure 13B).

Taken together our results showed that *expI* (AHLs synthase) and *hor* (*evf* regulation) genes have an important role in the *evf*-mediated development delay. Mutants in these two genes were unable to exert the delay in development as observed in the wt-infected larvae. Contrary to what was observed with *expI* and *hor*, *gacA* (response regulator from GacSA system) showed a partial phenotype. This result suggests that this second QS system also influences the *evf*-mediated phenotype but not as strongly as the other two genes. At the light of the current data, *evr* does not seem to have a role in the *evf* development mediated effect.

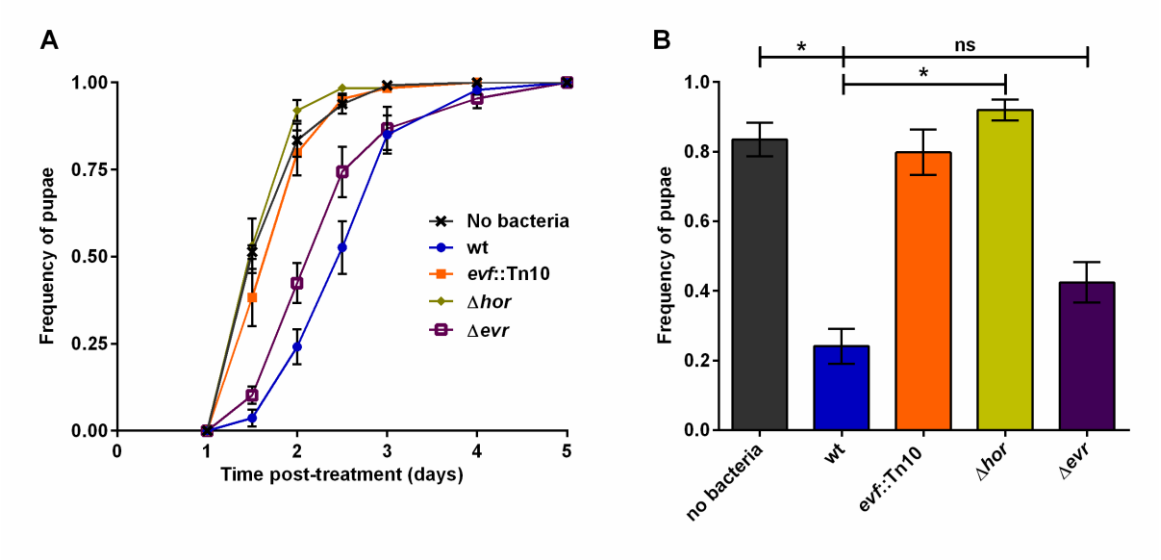

Figure 13 – Effect of *evf* regulator mutants on the development of *D. melanogaster* larvae. Larvae pupation frequency was evaluated by counting the number of larvae (32±7), in each independent replicate (n=5), reaching pupae stage after infection. Larvae were fed with standard fly food without bacteria as control, (dark grey crosses) or infected with Ecc15 (wt) (blue circles), *evf* mutant (orange squares), FDV57 (Δ*hor::kan*) (yellow diamonds) and FDV85 (Δ*evr::kan*) (purple open squares). (A) Time-course frequency of pupation after infection. (B) Represents the average pupation frequency of each treatment at day 2. Error bars represent the standard error of the mean, * stands for Pvalue<0.05 and ns for Not significant.

4. Discussion

In nature, *Drosophila melanogaster* is constantly exposed to microorganisms, relying on its innate immune system to survive. Some of these microorganisms, such as *Pseudomonas entomophila*[78], *Pseudomonas aeruginosa*[79] and *Pectobacterium carotovorum*[15] can induce immune responses with physiological consequences, when ingested by *Drosophila* larvae or adults. Ecc15 is a strain of *P. carotovorum* described as capable of persisting inside the gut of *Drosophila* larva[15]. In particular, it was found that persistence in the gut was associated with a single gene [60,71] named *evf* (*Erwinia* virulence factor). Additionally, two regulators of *evf* were also reported: Hor a protein from the SlyA family and Evr (*Erwinia* virulence regulator). It was shown in Ecc15 that Hor, acts as an activator of *evf*[60] but it is not known if the activation is direct or indirect. On the other hand, the role of *evr* was never assessed, although in a different strain of *P. carotovorum*[77] it is known to regulate the production of a cryptic pigment. Nevertheless, both the regulatory network of *evf* and its specific mechanism of action at the host level were poorly understood.

We hypothesized that *evf* expression is regulated by QS via RsmA, similarly to what occurs for the production of PCWDEs. *Pectobacterium* spp. relies on QS mechanisms to regulate the expression of virulence factors. Two major systems, ExpI/ExpR and GacSA/Rsm, regulate the expression of PCWDE, which are associated with plant infections. Mutants in these communication systems do not produce virulence factors, and are thus avirulent for plants.

Therefore, the overall aim of this work was to investigate the role of QS in the *evf*-mediated development delay as well as the *evf* specific mechanism of action. Moreover, we also investigated the network regulating *evf* transcription and the role of QS in this specific host-microbe interaction.

To address these questions we analyzed the developmental dynamics of *D. melanogaster* larvae infected with Ecc15. We have shown that wt-infected larvae display a development delay of approximately one day. We concluded that this development delay is *evf*-dependent, since larvae infected with a mutant in this gene do not present this phenotype. Supporting this result, larvae infected with Ecc15 overexpressing *evf* died without reaching pupa state.

Development delays are often associated with physiological changes such as nutrition. Closer observation of wt-infected larvae showed that they were thinner than those infected with the *evf* mutant. We therefore hypothesized that this development delay was caused by ceasing of feeding and to test this scenario we monitored food ingestion. Corroborating our

hypothesis, 3 hours post bacterial exposure larvae infected with wt Ecc15 had ingested a smaller amount of food compared to *evf* mutant-infected or control larvae. Our results also showed that this behavior was temporary; 22 hours post-treatment wt-infected larvae had resumed food consumption. These findings indicate that Ecc15 causes a development delay in larvae as result of a transient interruption of food ingestion and that this effect is somehow mediated by *evf*.

Although it has been hypothesized that Evf may interfere with the peristaltic movements of the gut[71], the specific mechanism that leads to this alteration of the normal physiology of the host was never assessed. Since we have shown that *evf* transiently changes the feeding behaviour of larvae and given that interruption of feeding is a common host response to bacterial infection[37,41], we hypothesized that Evf could be an extracellular toxin. We therefore tested supernatants from several bacterial cultures and a purified Evf protein in our larval development assays. Our results showed that neither the supernatants nor the purified Evf protein had a significant effect in *D. melanogaster* larvae. These findings do not support a possible toxic effect of Evf by secretion and strongly indicate that the presence of bacteria is needed to exert the *evf*-dependent effect on larvae. Despite these results, the possibility that *evf* codes for a toxin cannot be completely exclude. Secretion systems, present in several species of *Pectobacterium*, could allow the direct injection of Evf in the host cells[80,81], or alternatively targeting other bacterial species present in the fly natural microbiota. Type III and type VI secretion systems are the most promising systems to test. Type III injects bacterial compounds to host cells upon contact between bacteria and the host. These systems transfer bacterial proteins called effectors from the bacterial cytosol to the cytoplasm of the host cell, causing the disease[82,83]. These are used by several human and plant pathogens such as *Shigella* spp[84,85]., *Yersinia pestis*[86], *Dickeya dadantii*[87] or *Xanthomonas campestris*[88]. Type VI is used by bacteria to avoid competition in a polymicrobial environment. These systems have been identified in human opportunistic bacteria such as *Serratia marcescens*[89] and plant colonizers such as *Agrobacterium tumefaciens*[90]. In the case of Ecc15 infection, this system could be used to eliminate competitor species of the fly microbiota, thus creating space to colonize. One possible experiment that could be performed to prove this hypothesis would be to infect larvae with bacterial mutants in type III and type VI secretion systems and follow the development of those infected larvae. An alternative experiment to test type VI secretion systems could be infecting germ free flies with wt and *evf* mutant bacteria. If *evf* is attacking the fly microbiota, in a germ free fly both bacteria would cause the development delay. It is also possible that *evf* is not a toxin but its function is similar to the gene *ymt* from *Yersinia pestis*. In this human pathogen, acquisition of this gene changed its life style by allowing it to

colonize and survive in the gut of fleas[62]. After colonization this bacteria causes a blockage of the proventriculus (valve that connects esophagus and the midgut) which prevents food uptake and consequently fleas starved. It is possible that Evf function is similar to the *ymt* allowing colonization and survival in the midgut of *D. melanogaster* larvae. Transcriptomic studies in the context of the gut infection could reveal upregulation of genes associated with *evf* and thus highlight its role inside the gut.

We acknowledge that experiments testing persistence of this bacterial strain on the larval gut should be present in this work. Ecc15 is naturally resistant to ampicillin and thus we tried to plate wt- and *evf* mutant-infected larvae at several time points in LB+amp plates, to estimate the Ecc15 bacterial loads in the gut. Our results were inconclusive; we were unable to reproduce the obtained bacterial counts and most of the times we could not find plated bacteria. To our knowledge no studies were performed testing the minimal inhibitory concentration (MICs) of ampicillin in Ecc15. We therefore suspected that the ampicillin concentration used in the laboratory was too high for Ecc15 to tolerate. We have now optimized the proper antibiotics concentration and soon we will test persistence of Ecc15 bacteria inside the larvae gut.

As in the *Yersinia pestis* case, single acquisition of *evf* was associated with the ability of Ecc15 to colonize the gut of *D. melanogaster*. Even though single acquisition events are not new in the bacterial world[62], in order to be effective the genes acquired have to be integrated in the genetic network available in the bacteria. We hypothesized that *evf* transcription is QS-regulated by the same network that regulates production of PCWDEs. To test this, we constructed mutants in Ecc15 in both QS systems (*expI* and *gacA*) and *evf* known regulators (*hor* and *evr*). We characterized these mutants for plant virulence since *Pectobacterium* was originally described as plant pathogen. Our results reveal that, as previously described for other Pectobacterium species[19,91,92], mutants in cell-to-cell communications systems (*expI* and *gacA* mutants) were avirulent. Moreover, mutants in *hor* and *evr* genes also displayed impairments in plant virulence. Hor is a regulator protein found in many different bacterial species including human pathogens such as *Salmonella* and *Serratia*[61,93]. In *Salmonella*, this family of protein were found to regulate the production of haemolytic factors[94,95]. Additionally, in *Pectobacterium* was found to positively regulate the expression of the Car operon (carbapenem antibiotics)[61]. It is not surprising that mutants in this gene are impaired in virulence. It is possible that the production of antibiotics eliminates surrounding competition and consequently allows a better colonization of the potatoes or simply hor is regulating part of PCWDEs expression. Regarding *evr*, this gene was found to regulate the expression of a pigment in other strain of *Pectobacterium*[77]. Probably in Ecc15 *evr* is playing a role in the

adaption to the environment as it was described for other strains[77]. Interestingly, our results showed that *evf* is not required for plant virulence. In contrast to what was observed in larvae development, an *evf* mutant macerates potatoes the same way as the wt Ecc15. Since *evf* its not affecting potato maceration its role seems to be related only to the interaction with the fly which supports the theory of genetic acquisition. To further study the genetic network controlling *evf* transcription we analysed the GFP expression of both *hor* and *evr* promoter regions in these mutants. Our results indicate that QS might play a role in *evf* expression, since transcription of both *hor* and *evr* genes responded to the presence of AHLs. We also observed that at stationary phase, and contrasting with a typical QS regulation, expression is repressed by an unknown mechanism thus other factors are likely involved in regulating *evf* in liquid culture. We also concluded that *hor* and *evr* belong to two different networks since expression of one is not influenced by the other. We hypothesize that if both play a role in *evf* expression, then probably two different networks exists. This hypothesis can be tested by analysing the expression of *evf* over time, using a GFP reporter fusion, similarly to what was performed for *evr* and *hor* expression. Moreover, from these *in vitro* results, we exclude a possible role of *gacA* in regulating transcription of both *hor* and *evr,* since none of the promoter regions gene expression in this mutant was different from the wt bacteria. However, to fully clarify the network regulating *evf* transcription an analysis over time of its promoter region expression has to be done.

Although the experiments performed provide, indirectly, some information on *evf* genetic network we acknowledge that more data should be collected on *evf* expression. However, we could not obtain direct data on *evf* promoter region expression due to problems in the cloning process. Until now we were unable to successfully fuse the *evf* promoter region with the GFP tag, preventing the construction of the reporter plasmid. Additionally, there is a lack of genetic tools on this strain, and although it has been previously used in several studies it is not a common model organism.

Even though our *in vitro* tests did not provide enough information to fully understand the network controlling Evf production, they support the hypothesis that it is regulated by QS so we tested the role of QS in regulating the Evf effect *in vivo*. To prove this hypothesis we infected larvae with the QS and *evf* regulator mutants and evaluated their development. Our results revealed that the AHLs system plays a role in our *evf* mediated phenotype, since larvae infected with *expI* mutant did not show any development delay, behaving as the control larvae. However, we cannot confirm a direct role of QS in controlling *evf* transcription, since the absence of this QS system may also impair bacterial stress resistance necessary to face pressures from the host such as ROS and other chemical compounds. Analysis of the *evf*

promoter region will clarify the role of QS in this system. Nevertheless, it is clear that QS affects the *evf*-mediated effect either by changing Evf production or affecting the ability of the bacterium to survive inside the gut. In opposition, different results were obtained regarding the second QS system studied (GacSA/Rsm). Larvae infected with a *gacA* mutant showed a partial phenotype, displaying a developmental delay. This result contrasts with the analysis performed *in vitro* where we observed that *gacA* had no effect in the expression of both *hor* and *evr* genes. While it is true that we could not analyse the expression of the *evf* promoter region, the analysis of the expression of both *hor* and *evr* suggested that *gacA* was not a major contributor for the *evf*-dependent phenotype. However, the *in vivo* results showed that *gacA* as a partial effect being significant different from both wt-infected and control larvae which indicates that this gene plays a role in *evf* transcription or post-transcriptional regulation. It is also possible that, as in other bacteria, this communication system regulates other virulent factors that enable survival in the gut[78]. Recent results from our lab have shown that the GacSA/Rsm system affects positively the production of AHLs in *Pectobacterium wasabiae* (unpublished data). So it is also possible that the *gacA* mutant is affecting the production of AHLs and consequently the function of the AHLs QS system.

Regarding the *evf* regulation, our results also reinforced that *hor* is a primary regulator of *evf* as previously shown[60]. Larvae infected with this mutant did not display a development delay which indicates that in the absence of this regulator Evf production might be affected. On the other hand, larvae infected with *evr* mutant showed no significant difference to the wt. This result together with the *in vitro* results, here no kind of role was able to be determined, suggest that *evr* is not involved in *evf* regulation or its effect is weak.

Summarizing, we have shown that transient infection of Ecc15 is mediated by the *evf* gene and causes a development delay in *D. melanogaster* larvae. We have also showed that this delay is caused by ceasing of feeding and that the product of *evf* gene is not extracellularly active. We optimized a method to construct mutants in Ecc15 that allowed us to elucidate part of the genetic network involved in regulation of *evf.* Lastly we showed the role of the quorum sensing in the *evf*-mediated effect of Ecc15 in *Drosophila* development *in vivo*.

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6. Appendices

PCR protocol

Final volume= 25 µl

2.5 µl Dream taq Buffer (Fermentas)

0.25 µl DNTPs (100 mM)

0.25 µl Primers (100 mM)

1 µl template (100 ng/µl)

0.25 Dream taq polymerase (Fermentas)

Make up the 25 µl with miliQ H_2O

PCR conditions

5 min 95^oC ⟶ Hot start

30 cicles:

30 sec 95^oC ⟶ Denaturation

30 sec 60^oC ⟶ Annealing

1min (for each Kb) 72^oC ⟶ Extension

10 min 72^oC ⟶ Final extension

Final hold at 4^oC

PCR protocol with Bio-x-act proof reading

Final volume= 50 µl

2.5 µl optical Buffer (Bioline)

5 µl $MgCl_2$ (50mM) (Bioline)

0.25 µl DNTPs (100 mM)

0.25 µl Primers (100 mM)

1 µl template (100 ng/µl)

0.25 Bio-x-act (Bioline)

Make up the 50 µl with miliQ H_2O

T4 DNA ligase protocol

Final volume= 20 µl

100 ng/µl of vector

500 ng/µl of insert

1 µl of T4 DNA ligase (New England Bio)

2 µl of T4 DNA ligase Buffer (New England Bio)

Make up the 20 µl with miliQ H_2O

Incubate at room temperature for 2 to 3 hours

Electrocompetent cells protocol (glycerol method)

Day 1:

Do overnight cultures of the recipient strain (5 ml of LB + antibiotics if necessary) and incubate it at bacterial optimal growth temperature.

Day 2:

Add the 5 ml to 195 ml of LB and leave it to grow until OD=0.6 (Check OD every generation time).

Cool down the cells in ice for 20 min.

Divide in 4 50ml Falcon tubes and centrifuge at 4ºC, 4000 rpm for 20 min.

Discard the supernatant and resuspend the pellet using pipette (do not use Vortex) in 25 ml of ice cold Glycerol 10%.

Centrifuge at 4^{o}C 4000 rpm for 20 min.

Repeat the two previous steps 2 more times (total of three glycerol washes). The last wash can be done in 2 falcon tubes.

Discard supernatant and resuspend the pellet using pipette (do not use vortex) in the remaining 10% glycerol.